FINAL REPORT

Behavioral Ecology of Cetaceans: The Relationship of Body Condition with Behavior and Reproductive Success

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14. ABSTRACT

In this one-year limited-scope study, the objectives were to test our ability to make key measures needed to study the role of nutritive body condition in the behavior and reproduction in the Northern bottlenose whale (*Hyperoodon ampullatus)* and the humpback whale (*Megaptera novaeangliae*). Our approach was to collect animal-attached tag data, photographs, and tissue samples during three research trials in Canada and Norway and from opportunistic sources. Every one of the complex suite of measures was successfully carried out in at least one of the target species. Body density, the primary indicator of total body lipid store was successfully calculated from glides recorded by tags attached to three tagged bottlenose whales. We successfully measured progesterone levels in biopsy samples and detected it in non-invasively collected blow expirate samples to assess pregnancy status. This study demonstrated that it is feasible to conduct a multi-faceted study of body condition in free-ranging cetaceans.

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Behavioral ecology, cetaceans, body condition, behavior, humpback whales, bottlenose whales, biopsy, photogrammetry, hormone, lipid, body density

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LIST OF ACRONYMS

$3S^2$	$3S^2$: Behavioral Response Studies of Cetaceans to Navy Sonar Signals in Norwegian Waters
ANOVA	analysis of variance
API	atmospheric pressure ionization
ARTS	Aerial Rocket Tag System
BHT	butylhydroxytoluene
BW	Beaked whale
Cd	drag coefficient
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
cm	centimeter
d	glide depth
dB	decibel
DoD	Department of Defense
DTAG	digital acoustic recording tag
ELISA	enzyme linked immunosorbent assay
g	gram
GMT	Greenwich Mean Time
GSL	Gulf of St Lawrence
H	Humpback whale
HPLC	high-performance liquid chromatography
hr	hour
Hz	Hertz
KCl	potassium chloride
kg	kilogram
kHz	kilohertz
KW	killer whale
LC	liquid chromatography
LC-ESI-MS	liquid chromatography-electrospray ionization-mass spectrometer
LC-MS	liquid chromatography-mass spectrometry
LOD	limit of detection
M	meter
m	mass
m/s	meters per second
m/z	mass to charge ratio
MDC	minimum detectable concentration
MICS	Mingan Island Cetacean Study
Mm	millimeter
MV	Marine Vessel
Na_2SO_4	sodium sulfate
NB	Northern bottlenose whale
ng	nanogram
NS	not sampled
ONR	Office of Naval Research
PCAD	Population Consequences of Acoustic Disturbance
pg	picogram
r^2	coefficient of determination
rcf	relative centrifugal force

RMS	root mean square
SERDP	Strategic Environmental Research and Development Program
SI	Sustainable Infrastructure
SPE	solid phase extraction
SON	statement of need
TIC	total ion chromatogram
U	speed during glide
UK	United Kingdom
UPLC	ultra performance liquid chromatography
v	volt
VHF	very high frequency
ρ_{sw}	density of seawater
ρ_{whale}	body density
µl	microliter

KEYWORDS

Cetacean, body condition, lipid, body density, behavior, foraging, biologging, hormone analysis, biopsy, whale expirate, photogrammetry, *orca*, playback experiment, diving lung volume

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ABSTRACT

Objectives: The risk of harm to marine mammals from underwater noise is an important environmental and regulatory issue faced by the Department of Defense (DoD). In this one-year limited-scope study, the objectives were to test our ability to make key measures needed to study the role of body condition in the behavior and reproduction of toothed and baleen cetaceans, the Northern bottlenose whale (*Hyperoodon ampullatus)* and the humpback whale (*Megaptera novaeangliae*).

Technical Approach: Our approach was to collect animal-attached tag data, photographs, and tissue samples during three research trials in Canada and Norway and from opportunistic sources. The key analyses to be conducted in the study were carried out to evaluate our ability to perform the full study. The primary proposed indicator of total body lipid store was body density. This was measured via analysis of hydrodynamic performance when the animals were gliding (moving without active stroking). Measures of lipid concentration in blubber and body shape using laser photogrammetry were evaluated as possible approaches to cross-validate the tag-based measure of body density. Pregnancy status was evaluated using hormone analysis of progesterone, and nursing status was evaluated using observations of calves associated with subject whales. Tag data was used to describe foraging effort, and we conducted predator playbacks to simulate predator presence.

Results: Every one of the complex suite of measures was successfully carried out in at least one of the target species. Body density of three tagged bottlenose whales was successfully calculated. Insufficient time was available in the study period to also complete analyses of body density for humpbacks, but we were able to identify substantial levels of gliding and evaluate challenges to measure body density in this shallower-diver. Laser photogrammetry was conducted with both species, and our ability to measure some body dimensions was confirmed. However, girth is difficult to measure from a small boat, so alternative approaches to photographically assess body condition are proposed, particularly use of a high-resolution underwater scanning sonar. Lipid content in blubber samples was successfully measured in a number of sub-studies that evaluated variation in lipid content as a function of location on the body, depth in the blubber, and blubber thickness itself. We demonstrated our ability to measure lipid content of remotely collected biopsy samples, but variation by blubber depth and location on the body appears to differ by species. Biopsy tips of 60-100mm were found to be appropriate to measure lipid content across the entire blubber layer, but full blubber-depth biopsy samples are recommended to provide an estimate of blubber thickness at the sampling location. While neither photogrammetry nor lipid content in remotely-collected biopsy samples are themselves ideal estimators of total body lipid content, they are feasible to conduct and are potentially useful techniques to validate the estimate of total body density from analysis of glides recorded by tags. We successfully measured progesterone levels in biopsy samples and detected it within non-invasively collected blow expirate samples. Visual observations indicated none of our subjects were travelling with a calf. The high-resolution tags recorded a complex suite of measures that can be used to quantify foraging effort, energetic status, and anti-predator behavior. We successfully conducted playback of killer whale sounds to humpback whales.

Benefits: This limited-scope study was useful to demonstrate that, with some refinement in approach, it is indeed feasible to conduct all of the necessary components of a study of the role of body condition in the behavioral ecology of free-ranging cetaceans. While the work remains challenging, we suggest a two-tiered study focusing on role of body condition on the

foraging and anti-predator behavior of Northern bottlenose whales, and the role of body condition in the reproductive life history of the baleen humpback whale.

OBJECTIVES

The risk of harm to marine mammals from underwater noise is an important environmental and regulatory issue faced by the Department of Defense (DoD). Attempts to build defendable dose-response functions have been challenging in the face of limited data and variation in individual responsiveness. Long-term consequences of disturbance are particularly difficult to quantify. Noise may reduce foraging rates (Miller *et al*., 2009) and thereby nutritive *body condition* which is a good predictor of offspring survival (Hall *et al*., 2001) and reproductive success. Modeling exercises under the Office of Naval Research (ONR)-funded 'Population Consequences of Acoustic Disturbance' (PCAD) project have indicated that disturbance of foraging seals at sea could theoretically lead to population-level effects via effects on body condition. Body condition influences how animals trade-off foraging and anti-predator behaviors, and modulates responses to human disturbance (Beale & Mohaghan, 2004). Thus, behavioral ecology studies of how body condition relates to the risk and consequences of acoustic disturbance in cetaceans should be a high priority.

The objective of the full proposed research effort is to study the role of nutritive body condition (size of lipid store) on the foraging, anti-predator, and reproductive behavior of free-ranging cetaceans. The proposed work falls into two categories or phases of research. First is short-term intensive cross-sectional sampling of the condition, status and behavioral budget of individual animals within a population. During the initial stages of this first phase, we would seek to cross-validate body condition and reproductive state metrics using photogrammetry and biopsy sampling. Individuals will be repeat-sampled as much as possible over the duration of the study by focusing efforts on geographically faithful populations of animals. The second phase would be to link those short-term observations to long term survival and reproductive rates of identified individuals within a population. Survival and reproductive rates would be quantified using photo-identification re-sighting data and observation of calf presence. Our ultimate aim is to develop and ground-truth tools to the point that we can accomplish the research using non-invasive or minimally-invasive methods.

Success of the proposed research depends strongly upon successful application of tag-based methods to remotely measure the quantity of lipid fat carried in the total body stores of free-ranging individual cetaceans using analysis of underwater gliding performance. Analysis of gliding performance, specifically the rate of speed change during glides, enables derivation of total body density which is a good indicator of total fat stores in mammals (Biuw *et al*., 2003; Miller *et al*., 2004; Fields *et al*., 2005). Because there is no foreseeable means to validate body condition of free-ranging cetaceans using traditional methods such as isotope dilution (Reilly & Fedak, 1990), we would propose to conduct a set of measurements to cross-validate the tag-derived lipid content, including measures of lipid content in biopsy samples and photogrammetry measurements.

For the longer-term objectives that might be carried out in a full study, measures of body condition should be made simultaneous with observations of whether and how the quantity of lipid fat correlates with the subject's: 1) foraging effort and energetic performance and 2) anti-predator behaviors. For female subjects, fat store will be related to: 3) its reproductive status at the time (i.e., pregnant, nursing), 4) its reproductive output in subsequent years, and 5) survival of the subject and offspring in subsequent years when possible. As detailed below in BACKGROUND, patterns of lipid utilization are predicted to differ markedly between toothed and baleen whales. Therefore, the target species proposed for the study are the odontocete Ziphiid Northern bottlenose whale and the mysticete humpback whale

(*Megaptera novaeangliae*). Condition measures will be made of subjects of these species in controlled sonar exposure experiments being conducted in Norway under the ONR-funded $3S^2$ project (Principal Investigator: Miller), and body condition will be related to individual responsiveness to sonar as a anthropogenic stressor in those experiments.

Given the challenges of collecting and validating this complex suite of data with cetaceans, we first conducted a limited-scope effort to assess our ability to collect the samples, make the needed measurements, and identify appropriate field sites for the species to be studied. The specific objectives of the one-year limited-scope study were to evaluate our ability to:

1) Measure the quantity of lipid fat carried in the body stores by calculating body density of free-ranging individual Northern bottlenose and humpback whales using analysis of underwater gliding performance recorded on high-resolution tags.

2) Analyze photogrammetry and tissue biopsy samples in order to cross-validate the glide-derived measure of lipid fat content.

3) Use the high-resolution tag data to quantify that individual's foraging effort, energetic performance, and anti-predator behaviors.

4) Simulate predator presence via playback of killer whale calls.

5) Determine female subjects' reproductive status at the time as either pregnant, nursing, or resting. Assessment of pregnancy was accomplished using analysis of hormones (progesterone) collected in biopsy samples, and in blow expirate. Nursing cannot be observed directly, but was assessed by observation of calf association with tagged females at sea, and photogrammetry estimates of calf size.

6) Identify appropriate field sites to undertake the full study. This must take into account availability of animals, cost to reach the site, long-term study data (particularly photo-identification), and permitting requirements. For Northern bottlenose whales, it was foreseen that relating condition to life-history traits might be best accomplished in the Gully, but that method-validating research which will require biopsy sampling may be best accomplished in Norwegian waters. For humpback whales, the Gulf of St Lawrence field site offers the potential for shore-based research combined with the long-term humpback-population research program of the Mingan Island Cetacean Study (MICS) (Ramp *et al*., 2010).

BACKGROUND

This proposal responds to the Strategic Environmental Research and Development Program (SERDP) 2011 request for proposals 'Behavioral Ecology of Cetaceans'. One of the important predicted impacts of disturbance is that affected animals will be unable to feed effectively, and may expend more energy to relocate and find food (Miller *et al*., 2009). To lead to a population-level impact, such effects should feed through to effects on reproductive and survival rates. For marine mammals the quantity of the fat energy store is an intermediate parameter that integrates such impacts with the foraging efforts of individuals, and ultimately should also be a good predictor of the reproductive health of populations. In phocid grey seals, Hall *et al*. (2001) demonstrated that fat stores in the mother was an important predictor of the chances of first-year survival in her offspring. Because cetacean mothers also face extensive gestation and lactation demands, body stores are expected to by closely tied to reproductive cycles and ultimately to reproductive rates and adult and offspring survival.

Body Condition in Behavioral Ecology Research

Body condition has been demonstrated to be a predictor of fitness in many ways including survival and reproductive success (Moya-Laraño, 2008; Bradford, 2012). Body condition can be defined in numerous ways, but is usually measured as nutritive condition, specifically the quantity of energy stores carried in the blubber and other fat stores of an individual. The size of those stores reflects an integration of foraging effort and foraging success, but also reflects the reproductive needs of the individual in their life cycle. For example, for seals that breed on land, researchers have been able to quantify the resources required to produce offspring and the consequences of variation in resources on future survival and reproductive success (Pomeroy *et al*, 1999; McMahon *et al*. 2000). Capital-breeding seals, such as the British grey seal (*Halichoerus grypus*), fast while breeding, and female fat stores predict reproductive success (Hall *et al*., 2001).

In addition to potentially being a good indicator of the reproductive health of populations, body condition may be an important predictor of behavior patterns in cetaceans. Evolutionary theory suggests that fitness can be optimized if organisms vary responses to the environment dependent on their status or condition (Rands *et al*., 2004). Biological theory predicts that animals in poorer nutritive condition should increase their foraging efforts, including increasing the risk of being predated upon, in order to increase their foraging success (Frid & Dill, 2002). Body condition is predicted to influence how individuals trade-off foraging and anti-predator behaviors (McNamara and Houston, 1990; Hilton *et al*., 1999), with poor condition foragers taking greater risks in order to increase foraging rates. This leads to two important predictions. The first is that cetaceans should have some ability to adjust their foraging effort to recover their desired body condition, or to maintain "allostasis" (McEwen and Wingfield, 2003). Thus, individuals whose body condition is poor, including any that might have been negatively affected by human disturbance, are predicted to have some capacity to compensate for those effects, but the effort of compensation could have its own costs, such as increased predation risk or delayed reproduction. The second prediction is that animals in poor body condition should be more tolerant of disturbance sources than animals in good body condition, a prediction which has been tested and confirmed in birds (Beale & Mohaghan, 2004).

Our full proposed work would seek to test these predictions with the Northern Bottlenose whale and the humpback whale. The baleen mysticete humpback whale is one of the better

studied baleen whales, with numerous studies on their life-history and behavior (Clapham, 2000). Humpbacks undertake an annual fasting cycle as part of their migration to breeding grounds. Thus, as for phocid seals, deposition and utilization of lipids are expected to occur on an annual cycle, and the amount of lipid stored during the feeding season should be an important factor in shaping their behavior and reproductive success. Patterns of lipid utilization are expected to differ markedly from that of toothed whales that are thought to feed year round.

Body Condition in Free-Ranging Cetaceans

The proposed tag-based method for estimating body density (Aoki *et al*., 2011) provides a novel technique for establishing the full-body lipid content body condition of free-ranging cetaceans in a replicable and quantitative fashion. Because lipids are less dense than other animal tissues, one of the consequences of changes in lipid stores is a change in body density, which can be used to estimate lipid content (Biuw *et al*., 2003). Indeed, body density measured via 'plethysmography' is the state-of-the-art method to measure body condition of humans (Fields *et al*., 2005). Body density can be measured directly by analysis of performance during glides, which are periods in which the animal is not actively producing thrust with its swimming organs. Glides can be identified unequivocally using accelerometers to register animal thrusting movements (Sato *et al*. 2003). Gliding during descent and ascent phases of dives is a common behavior that increases when movement is aided by buoyancy (Williams *et al*. 2000; Miller *et al*. 2004). Sato *et al*. (2003) showed that percentage of time gliding, and ascent and descent rates of diving Weddell seals was strongly correlated to girth/length fatness ratios.

Ultimately, however, % time gliding and ascent and descent rates are behavioral decisions made by divers that can be affected by factors other than lipid content, such a motivational state. The method of directly calculating body density from glides removes behavioral variation as the speed and acceleration of gliding bodies are a predictable outcome of the specific drag and buoyancy forces that act upon them. Body density reflects the state of the total body fat store, wherever in the body lipids might be located, and thereby would represent a substantial improvement to existing methods, reviewed below.

Three different methods exist to estimate body density from glides. The drift dive method (Biuw *et al*., 2003) requires animals to drift motionless at depth – a behavior limited to few species. The all-glide method makes use of all available glides to fit a three term model of drag, air buoyancy, and tissue buoyancy and has been successfully used with digital acoustic recording tags (DTAGs) on sperm whales (Miller *et al*., 2004). The prolonged glide model (Watanabe *et al*., 2006) estimates body density from the terminal descent gliding velocity. Research in the Miller lab has validated these methods with elephant seals where fat content was measured using doubly-labeled water (see Biuw *et al.*, 2003 for details). Three elephant seals were outfitted with Little Leonardo 3MPD3GT tags, and detachable weights and floats to manipulate their body density. All three methods gave a good correlation (r^2 0.95-0.99) with density estimated from fat content derived from labeled water measurements (Figure 1; Aoki *et al*., 2011).

Work on blubber-thickness in the Northern right whale indicates that that blubber stores vary strongly with reproductive status (Moore *et al*., 2001; Miller *et al*., 2011). Similarly, photogrammetry-based visual assessment of body condition indicated that gray whales with calves had worse body condition than females generally, though calf condition appeared to be uniformly good (Bradford *et al*., 2012). A drawback of the work-to-date with cetaceans is

that it remains unclear how metrics such as blubber thickness at one location and visually-assessed-condition relate to the true status of the underlying lipid store. However, photogrammetry and blubber analyses are the state-of-the-art techniques to cross-validate the novel tag-based metric proposed in this study.

As recently discussed by Bradford *et al*., (2012), most of the methods commonly used to measure body condition of terrestrial mammals cannot be accomplished with free-ranging cetaceans. Photogrammetry, the measurement of body size or shape from photographic images provides a possible alternative. However, measuring whales photographically at sea is challenging as only part of the body is visible and for only a short amount of time. Estimation of full body length therefore often requires understanding of allometric relationships of certain body parts (e.g., fluke width, blowhole to dorsal fin distance, dorsal fin height or width) usually based on data from captive or stranded animals (e.g., Clark & Odell 1999; Sousa-Lima & Groch 2010).

Some of the earliest photogrammetric studies of cetaceans investigated the relative proportion of certain body regions rather than absolute measurements (e.g., using height-width ratios of the dorsal fin to sex killer whales, *Orcinus* orca; Heimlich-Boran 1986). The use of calibrated cameras and range estimation (usually using laser range-finders) allowed the first determination of absolute measurements of body dimensions from photographs (e.g., Jaquet 2006; Sousa-Lima & Groch 2010). However, the calibration of cameras and lenses is time-intensive and precludes the use of zoom lenses, which can very useful for smaller cetacean species.

Laser photogrammetry does not require calibration of photographic equipment, and instead uses two parallel lasers mounted on a digital camera to establish scale. The lasers project dots at a known distance apart onto the animal photographed, and thus allow absolute measurement of different body parts. Laser photogrammetry has been used successful on killer whales (Durban & Parsons 2006) bottlenose dolphins (*Tursiops truncatus*; Rowe *et al.* 2010) and Hector's dolphins (*Cephalorhynchus hectori*; Webster *et al.* 2010)

Aerial photogrammetry relies on photographs of cetaceans at the water surface taken from above (usually from an airplane) at a known height. Height is usually established using a radar altimeter while some form of level ensures that the camera points straight down (Mocklin *et al.* 2010). Because aerial photogrammetry requires use of an airplane and expensive photographic equipment, this method is comparatively costly. However, it does have the advantage the entire body of the animal can be measured from the photograph, and therefore information about allometric relationships is not necessary. Aerial photogrammetry has been used successfully on several cetacean species (e.g., killer whales, Pitman *et al.* 2007; Fearnbach *et al.* 2011) and is the only method so far that has been used to successfully measure body condition in cetaceans (Perryman & Lynn 2002)

Stereo-photogrammetry allows the three dimensional measurement of objects by interpreting pairs of photographs taken simultaneously from different angles. Several studies have used cameras mounted on a research vessel a fixed distance apart and synchronized electronically to measure body size of cetaceans at sea (e.g., Bräger & Chong 1999; Bräger *et al.* 1999; Chong & Schneider 2001). While compared to laser photogrammetry the apparatus is comparatively unwieldy, stereo-photogrammetry does have the advantage that it allows measurements in three dimensions (rather than just in the plane parallel to the boat) making method potentially valuable for studies of body condition.

Three-dimensional photogrammetry is an extension of stereo-photogrammetry where multiple photographs are taken of a quasi-stationary subject from a variety of angles. If the photographs can be referenced to stationary objects in the environment, this method allows three-dimensional modeling of the subject with high precision. While three-dimensional photogrammetry has been used very successfully on hauled-out pinnipeds (including the assessment of body weight and body condition; de Bruyn *et al.* 2009), its application to cetaceans is probably limited because these animals are hardly ever stationary for any length of time and because stationary reference points are not available at sea. However, applying some of the photographic modeling software developed for three-dimensional photogrammetry on data collected using other methods may hold potential.

An alternative approach to cross-validate body condition of cetaceans is to explore techniques to remotely measure lipid content within the blubber, as previous research has shown that total blubber lipid is a reliable indicator of total body fat (Evans *et al*. 2003, Thiemann *et al*. 2006, Gomez-Campos *et al*. 2011), the animals' principle energy store, which in turn defines nutritive body condition. Total body lipid content can be simplified as a function of blubber thickness (Moore *et al*., 2001) and lipid content within the blubber (Aguilar & Borrell, 1990). However, the distribution of this energy store within the blubber layer can vary by depth in the blubber and body location as well as season, sex and reproductive status (Olsen & Grahl-Nielsen 2003, Samuel & Worthy 2004, Gomez-Campos *et al*. 2011). A major effort of this Limited-Scope study has been to evaluate the potential for using measures of lipid content within remotely-collected blubber biopsy samples to estimate nutritive body condition.

MATERIALS AND METHODS

Field Trials

In this Limited-Scope study, our overall methodological approach was to evaluate all different components of the proposed research, in order to reduce the risk of accomplishing the full outlined study. Data were collected in three research trials (Figure 1; Table 1), using either the Little Leonardo 3MPD3GT logger or the DTAG (Table 2).

Figure 1. *Maps indicating the three field sites – Gulf of St Lawrence, The Gully and S.W of Spitsbergen.*

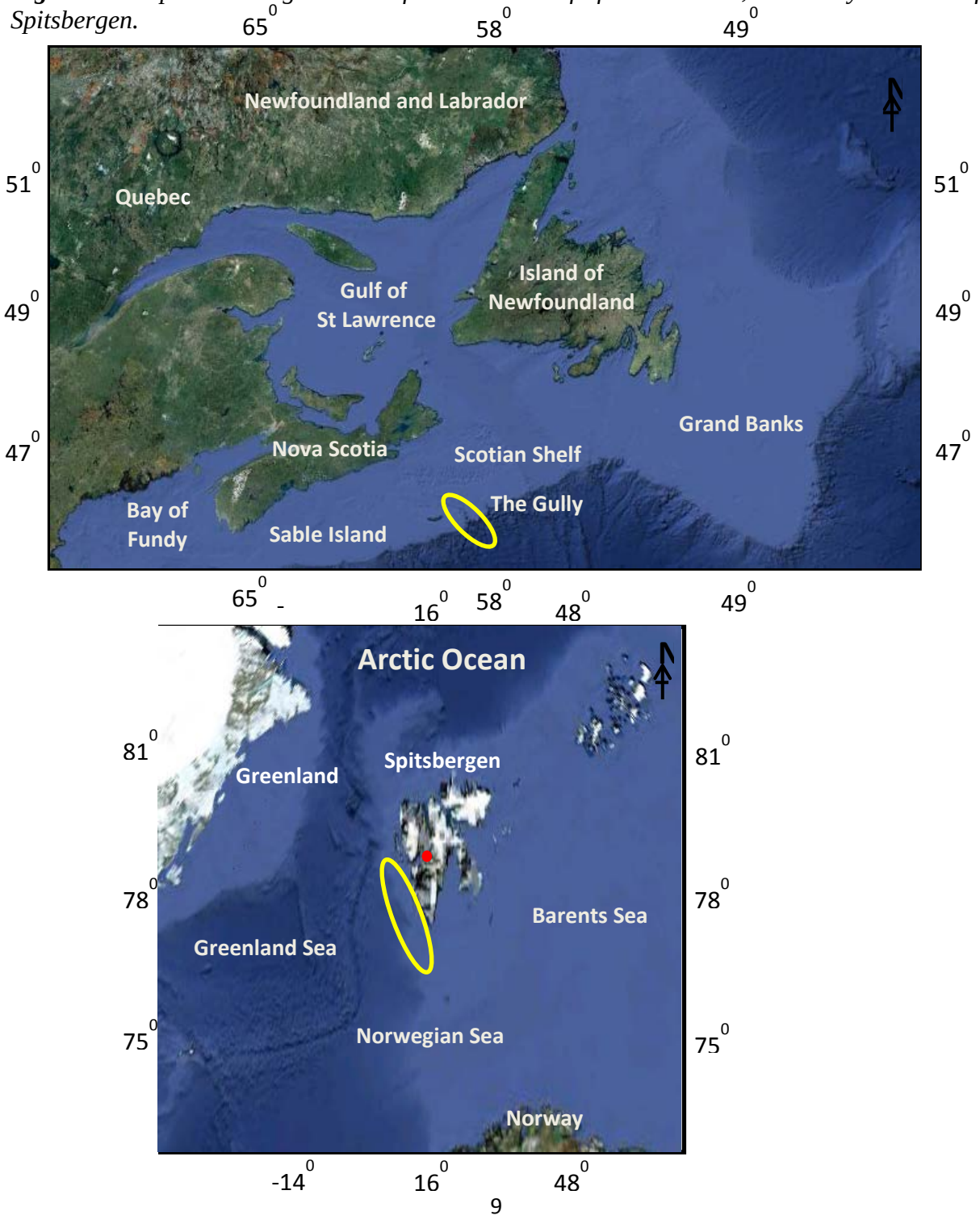


***Table 1**. List of fieldwork activities undertaken for the two target species humpback whales (H) and Northern bottlenose whales (NB) in the three field sites. Note that blubber samples were also obtained from stranded cetaceans, including the Northern bottlenose whale.*

Field Site:	Tagging	Biopsy	Expirate	Photo	orca
Gully	NB	-	NB	NB	-
Gulf of Saint Lawrence	H	H	H	H	-
Norway	H	H	-	H	H
Stranding	-	NB +	-	-	-

One SERDP-supported pilot trial was conducted in the Gulf of St. Lawrence (GSL) from mid-July to mid-September and focused on the humpback whale as a target species and used the Little Leonardo 3MPD3GT as the primary tag tool. A second 10-day SERDP-supported pilot trial was conducted in the Gully Canada and focused on the Northern bottlenose whale again using the 3MPD3GT. Supporting measurements and methods trials were conducted during a third 30-day research trial funded by ONR on the effects of sonar on cetaceans in Norway with tagging utilizing the version two DTAG. In addition, research material for blubber tissue analyses were obtained from stranded or by-caught cetaceans through the United Kingdom (UK) stranding-network scheme. Finally, one 3MPD3GT tag record of a Cuvier's beaked whale tagged off Japan was provided by the University of Tokyo for analysis of body density.

***Table 2**. Sampling rate of key parameters measured by the two loggers used in the study.*

	Depth	Speed	Temp.	3-axis acceleration	Acoustics
3MPD3GT	1 Hz	1 Hz	1 Hz	32 Hz	---
DTAG	50 Hz	---	50 Hz	50 Hz	192 kHz
Use in the study	Gas compression, speed proxy	Acceleration and speed of glides	Estimate ρ_{sw} at depth	Define gliding periods, measure animal pitch	Foraging effort, flow noise as speed proxy

During the 10-day trial in the Gully with bottlenose whales, weather was un-seasonally windy for August, and only three of the eight days on site in the Gully were workable for approaching whales (Figure 2). On those three days, three bottlenose whales were successfully tagged and the instruments were recovered, indicating that this species is a relatively easy-to-tag member of the family Ziphiidae. In addition three blow-expirate samples were collected and over 1800 identification photographs were taken, including photogrammetry images. Observations of group-level behavior indicate that none of the tagged animals were travelling with a calf. We did obtain permits to collect biopsy samples

and to conduct orca playbacks, to this species which is listed 'at-risk' and the research is also within a Marine Protected Area. However, no biopsy samples were collected as the permit stipulated a tip-length (60mm) which was unavailable in time for the trial. With permission granted for all activities in 2011, we consider the ongoing risk for obtaining permissions to conduct the research to be low. No *Orca* playbacks were conducted due to poor tracking of the tagged whales as the tags were placed rather low on the body. The tag-placement problem can be easily resolved by using a 90° attachment system to allow the tags to be attached higher up on the body.

Figure 2. *A Northern bottlenose whale near the Marine Vessel (MV) 'ON A MISSION' used in this project within the Gully, Canada. This species was easily encountered and approached for research in the Gully Marine Protected Area. Photo by Volker Deecke.*

During the two-month trial in the Gulf of St Lawrence with humpback whales, it was possible to conduct a relatively low-cost shore-based effort with working days on the water during good weather. During this period, 12 humpback whales were tagged, one whale ('Meduse') was tagged twice. All of the tagged whales were photo-identified, and observations of group structure were made successfully – none of the tagged whales were travelling with a calf. Supporting photogrammetry images were made of most of the tagged whales. A total of nine biopsy samples were made of the tagged whales using a 20mm dart, and blow samples were collected concurrently in seven cases. No *Orca* playbacks were conducted as these were not requested in the permit. *Orca* sound playbacks were trialed instead in the 3S trial funded by ONR.

The 3S trial lasted 30 days and was focused upon studying the reactions of humpback, minke, and Northern bottlenose whales to 1-2 kHz sonar signals (Kvadsheim et al., 2011). Playback of *Orca* sounds to tagged whales has been a routine part of the 3S protocol since the initial playbacks were conducted in 2008. Playbacks of *Orca* and noise control sounds were conducted successfully to three tagged humpback whales. The 3S project board agreed to support the body condition project, and a dedicated effort was made to collect biopsy samples from tagged humpback whales. A total of seven humpback whales were tagged with DTAGs of which three were successfully biopsy sampled, two with a 100mm dart length and one with a 40mm dart length. These biopsy samples were collected using an Aerial Rocket Tag System (ARTS) air-compression system capable of launching a larger projectile than the cross-bow system used in the GSL. Due to time constraints and the intense schedule, it was not possible to either collect blow-expirate samples or deploy 3MPD3GT loggers during the Norway trial. While Northern bottlenose whales were encountered on two days during the trial, there was insufficient time to place a tag or to collect other samples.

Stranded Cetacean Blubber Samples

Blubber samples and accompanying information on species, sex, body length, age and cause of death, were collected from stranded animals by the UK Cetacean Strandings Investigation Program. Staff at the Scottish Agricultural College, Inverness, were responsible for blubber sample collection from the stranded animals. Approximately 2.5cm^2 to 5cm^2 blocks of full depth blubber were collected from between 12 and 18 separate sites across the right side of the body of five individual odontocetes of different species and were frozen prior to analysis.

DETAILED METHODS, RESULTS AND DISCUSSION

In this section, we consider the core data-analysis efforts of relevance to the Limited-Scope effort. These are broken down as listed in the OBJECTIVES section, and separate detailed methods, results and brief discussion are given for each section. The focus of the data analyses is to evaluate our ability to succeed in measuring the specific parameters of interest, and to consider how results reduce risk for success in the overall project goals.

1) Measure Body Density Using Glides Recorded on Tags

Measure the quantity of lipid (fat) carried in the body stores of free-ranging individual Ziphiid Northern bottlenose and humpback whales using analysis of underwater gliding performance recorded on high-resolution tags.

Key Findings: Body density of Northern bottlenose (and a Cuvier's beaked whale) and humpback whales calculated from high-resolution tag data.

- Body density analysis of Northern Bottlenose whale and Cuvier's beaked whale was conducted successfully for all deep dives obtained using the all-glide method. Numerous appropriate glides were identified during both descent and ascent phases, indicating that the method can be applied to the taxonomic group Ziphiidae.
- The results indicate a body density that is somewhat negatively buoyant, with all individuals maintaining body density within a narrow range with differences between individuals of less than 0.01%. This likely suggests that these toothed whales maintain a narrow range of body densities via allostasis.
- The analysis of Humpback whales is in a much earlier stage, but we have identified that they do make use of glides in their behavior. Percentage of time gliding during ascent and descent changed for one animal that tagged both early and then later in the feeding season, but the changes do not clearly indicate an increase in fat store as was indicated from the blubber biopsy analysis of the same whale. The analysis approach to estimate body density for humpback whales appears more challenging than that for bottlenose whales and may need to take into account diving air volume for each dive.
- For the three Northern bottlenose whales and one Cuvier's beaked whale, we were also able to measure the diving lung volume. While diving lung volume may not influence body density, per se, and might be ignored for deep divers such as the beaked whales, it may be a critical consideration for relatively shallower-diving animals such as the humpback whales. Our analysis demonstrates a joint analysis of body density and diving lung volume is potentially feasible.

1.1 Body-Density and Diving Lung Volume of Northern Bottlenose Whales and a Cuvier's Beaked Whale from High-Resolution Tag Data.

Specific Detailed Methods

During fieldwork in the Gully, the underwater canyon in the Atlantic Ocean, from August 1 – 12, 2011 we deployed a multi-sensor data logger (W2000-3MPD3GT; Little Leonardo Co., Tokyo, Japan) to three bottlenose whales (Table 3). W2000-3MPD3GT logger recorded three-axis magnetism, swim speed, depth and temperature at 1Hz, and three-axis acceleration at 32 Hz. In addition data from a single Cuvier's beaked whale, using the same tag device, was made available by Dr Kagari Aoki and colleagues.

Table 3. *Summary of Ziphius data used to estimate body density.*

Tag date	Tag ID	Tag on	Tag off	Duration (hours)
07 August	ha11-218a	9:30 local	18:17	8.2
08 August	ha11-219a	9:19	11:45	2.5
11 August	ha11_222a	7:44	15:24	7.7
14 September 2010	Cuvier's BW	10:49	8:30 (15 Sept)	21.6

Cd*A/m and body density (ρ_{whale}) were estimated by the glide model (Aoki *et al*., 2011). The simple form of this equation is:

$$\text{Acceleration} = -0.5*(Cd*A/m)*\rho_{sw}*U^2 + (\rho_{sw}/\rho_{whale} - 1)*g*\sin(\text{pitch}). \quad (1)$$

Acceleration is the change of speed during the glide, Cd is the drag coefficient, A is the reference surface area, m is the mass of the animal, ρ_{sw} is the density of seawater, U is the speed during the glide, ρ_{whale} is the density of the whale, g is the gravitational constant, and pitch is the vertical angle of the whale during the glide. This equation relates the change in speed to the combination of drag (left part of equation) and body buoyancy (right hand side) forces. Note also that in this equation the influence of gases is ignored (Miller *et al*., 2004), which is acceptable for the deep glides used in this analysis (Aoki *et al*., 2011).

All glides in the tag deployment periods were detected using the amplitude of signals on the accelerometers. Glides were divided into 5-s sub-glides. Sub-glides used for this analysis had to meet the following criteria:

1. descent & ascent glides during deep dives (max depth > 1000m)
2. depth > 200m
3. |pitch| > 30 degree

The depth criterion is required as the effects of gases are being ignored. Relatively steep pitch glides are used as these are most strongly affected by buoyancy as in equation one.

The density of seawater (ρ_{sw}) is known to change with pressure, temperature, and to some extent salinity. The simple form of the model above assumes that the density of the whale is constant independent of dive depth, which is not particularly realistic. To account for changes in density with depth, two other forms of the equation were evaluated. Model two assumed that the ratio of whale to seawater density is constant. Model 3 assumes that the ratio of whale to seawater density is not changed by depth (as pressure acts similarly upon seawater and the whale body), but that temperature effects will not influence the body density of the homeothermic whale. Model 3 can be written as:

$$\text{Acceleration} = -0.5*(Cd*A/m)*\rho_{sw}*U^2 + ((\rho_{sw} + \Delta\rho_{sw}(t, d))/ \rho_{whale} - 1)*g*\sin(\text{pitch}) \quad (2)$$

where $\Delta\rho_{sw}(t, d)$ is a correction factor that equals the difference between the density of seawater at the temperature and depth of the glide and the density of seawater at the depth of the glide but at a reference temperature taken at 1m depth. This correction is the same as was

used in Miller *et al*. (2004, eq. five). In the following analyses, Models one and three were used. Model two was evaluated, but results are not shown here as Model 3 was preferred.

Lastly, a model was applied to the data to further estimate the diving lung volume of the whales using the equation:

$$\text{Acceleration} = -0.5*(Cd*A/m)*\rho_{sw}*U^2 + ((\rho_{sw} + \Delta\rho_{sw}(t, d))/ \rho_{whale} - 1)*g*\sin(\text{pitch}) + (Vair(0)/m)*g*\sin(\text{pitch})*[\rho_{sw} - \rho_{air}(1 + 0.1*d)]*1/(1 + 0.1*d) \quad (3)$$

In which Model 3 is used plus a term for the buoyancy effect due to volume of air gases carried by the body (Vair), which was referenced to volume at the surface, but changed with glide depth following Boyle's Law (Miller *et al*., 2004). ρ_{air} .is the density of gas carried by the whale, which also changes as a function of glide depth (d). For air-volume analyses, glides across all depths were used. All model fits were carried out using non-linear least squares with JMP statistical software.

Results and Outcomes

The Northern bottlenose whales in this study made steep dives, both during dive ascent and descent (see Table 4). Thus, there were a very large number of glides available for each dive from which to estimate the parameters in the model. As was detailed above, the fit for Model 3 was substantially better than for Model 1 (Figure 3), and the residuals versus depth are flat for Model 3 unlike Model 1 (Figure 4). This demonstrates the importance of accounting for temperature effects on seawater density at depth.

Body density of the tagged *Ziphius* and *Hyperoodon* measured from glides >200m deep ranged from 1029.8 ± 0.1 to 1031.8 ± 0.1 kg/m^3, indicating negative buoyancy in sea-water and little variation between individuals in our sample (Table 4). Pooling all glides from deep dives (>1000m) by individual, air volume of the *Hyperoodon* ranged from 15.5 ± 6.2 ml/g to 22.6 ± 1.8 ml/kg and the *Ziphius* was 20.8 ± 2.5 ml/kg.

Table 4*. Estimated parameters for beaked whales with 95% confidence intervals shown in brackets. The parameters (Cd A/m) are estimated jointly, ρ_{whale} is the body density of the whale, and Vair is the diving lung volume of each whale. Note the large number of glides available for the analysis.*

Tag ID	Cd A / m [95% CI]	ρ_{whale} (kg/m^3)	Vair (liters)	# glides
ha11-218a	0.0000177	1031.80	22.6	303
	[0.0000171, 0.0000183]	[1031.67, 1031.94]	[20.8, 24.4]	
ha11-219a	0.0000149	1030.81	17.0	148
	[0.0000143, 0.0000156]	[1030.67, 1030.95]	[12.6, 21.4]	
ha11_222a	0.0000162	1030.99	15.5	243
	[0.0000147, 0.0000176]	[1030.67, 1031.31]	[9.2, 21.7]	
Cuvier's BW	0.0000228	1029.80	20.8	527
	[0 .0000234,0.0000222]	[1 029.71,1029.89]	[18.3 23.3]	

Figure 3. *Relationship between acceleration (measured and predicted) and swim speed. Colors of measured acceleration shows pitch angles. Black markers in the left figures indicate predicted acceleration under Model 1, and the green circle in the right figures indicate predicted acceleration under Model 3. Measured accelerations are colored based upon the pitch during the glide, with dark blue representing steep descent glides and dark red representing steep ascent glides. Note the superior performance of Model 3 in reducing the variation in the predicted acceleration during glides.*

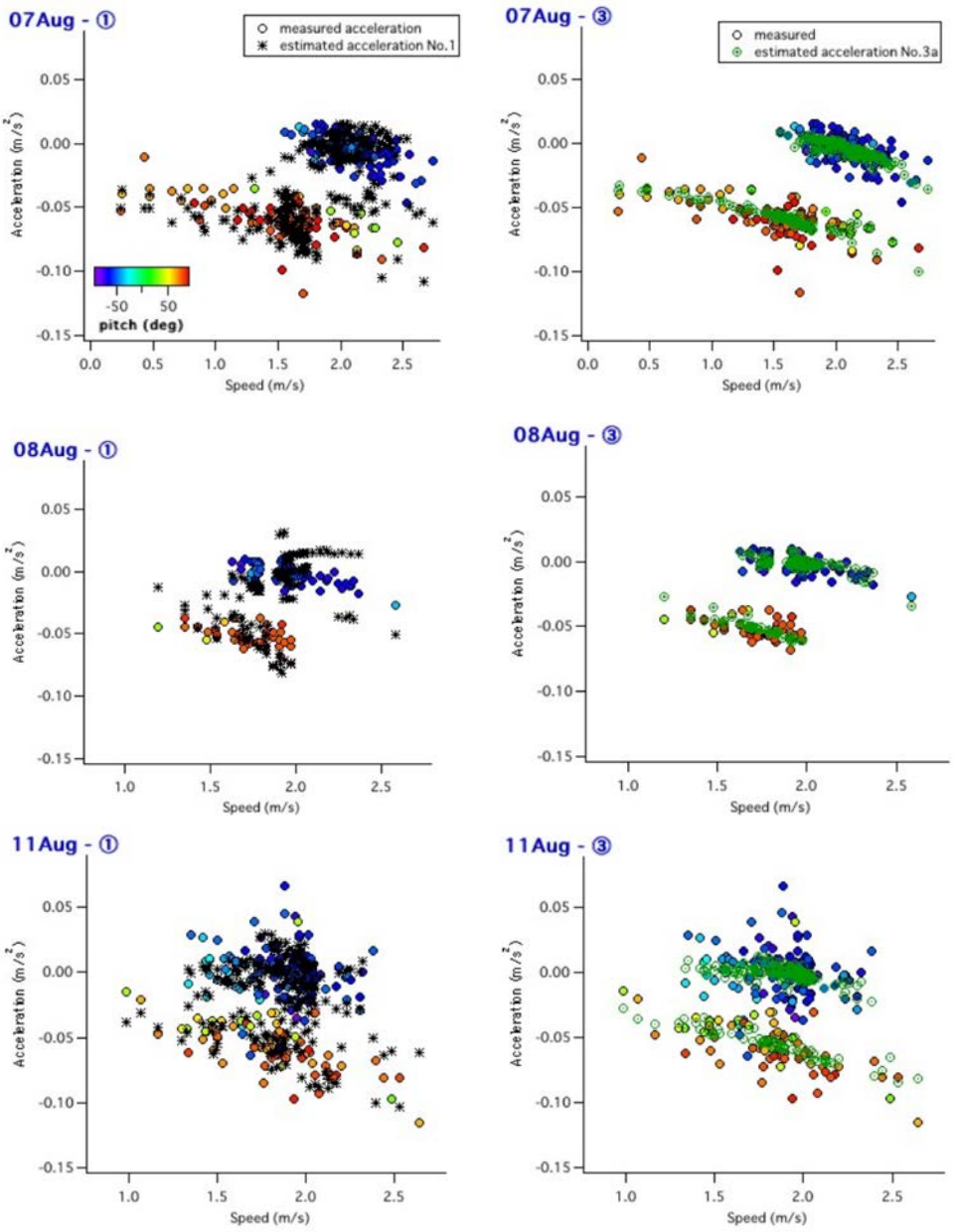
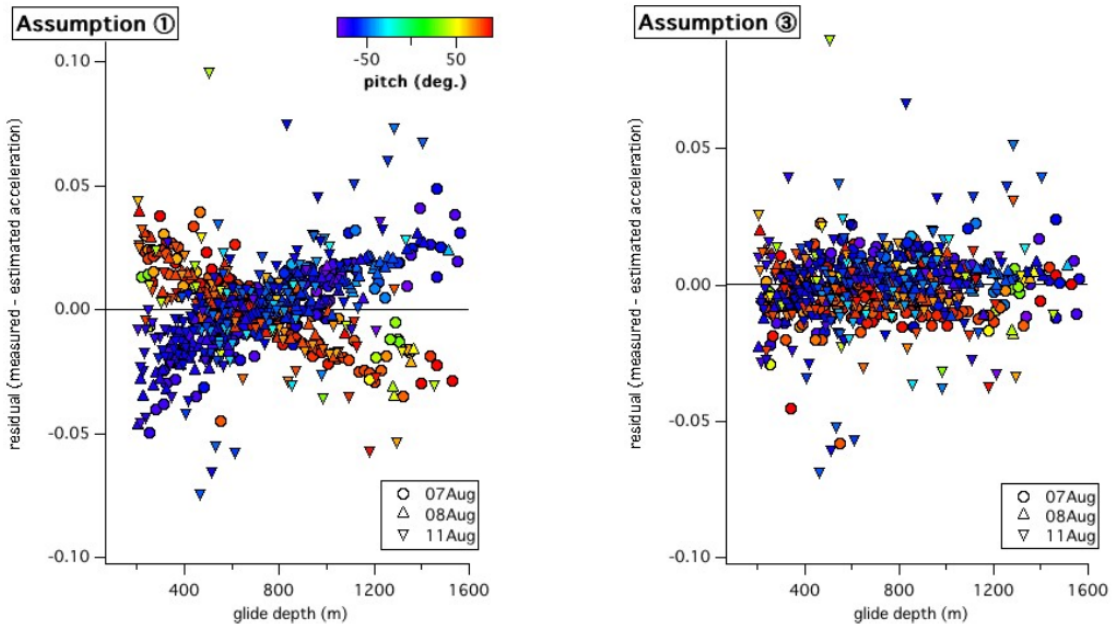


Figure 4. *The pattern of residuals of the two different models. Notice that Model 1 has patterned residuals against glide depth, as expected due to the lack of a temperature correction term. Model 3 has flat residuals versus depth.*



1.2 Body-Density Estimates of the Humpback Whale – Initial Efforts

Given the short duration of this Limited-Scope study, we directed our tag data analysis efforts to fully completing the relevant analyses of body density for the bottlenose whales and additional Cuvier's beaked whale. As a result, little time has been available to attempt to measure the body density of humpback whales. With the limited time available, we inspected in more detail the diving and gliding behavior of the one individual that we tagged twice during the feeding season ('Meduse').

Specified Detailed Methods

Data of humpback whales were collected during July and September 2011. In this study, W2000-3MPD3GT was used to record three-axis magnetism, swim speed, depth and temperature at 1Hz, and three-axis accelerations at 32 Hz (Table 5). Data was inspected in detail using IGOR PRO. Glide periods were identified in the dive records based upon the amplitude of oscillations on the accelerometer.

Results and Outcomes

The data images below show time-series data obtained from Meduse0722 and Meduse0911. As can be clearly seen in the data series, Meduse0722 (Figure 5) conducted many shallow dives (<100m), most of which were associated with lunge feeding (see Figure 6 for example). On the other hand, Meduse0901 (Figure 7) conducted some deep dives (> 100 m) which contained glides appropriate for the analysis of body density (Figure 8).

For this animal, we conducted an initial analysis of the time spent gliding in ascent and descent phases, which has been correlated with body girth in Weddell seals (Sato *et al*., 2003). If Meduse added substantial lipid from 22 July to 01 Sept, we might expect gliding rates during the descent phase to decrease and gliding rates during ascent phase to increase. Instead, the analysis (Table 6) indicated that the % of time gliding was highly variable overall and was affected by whether or not dives contained feeding lunges. There is no clear body condition signal using %-of-time gliding alone.

Table 5. *List of details of tag deployments on humpback whales in the Gulf of St Lawrence. Note that the animal Meduse was tagged on two separate occasions.*

Date	Animal	Name	Species	Sex	Biopsy	Blow Sample	Tag #
2011-07-21	H584	Manta	Mn	F			03745
2011-07-22	H607	Meduse	Mn	M	CAR11014	yes	03745
2011-07-25	H671	Pythagore	Mn	M	CAR11015	yes	87666
2011-07-25	H686	Not named	Mn	F	CAR11016	no	03745
2011-07-26	H698	Not named	Mn	M	CAR11017	yes	87666
2011-07-26	H731	Not named	Mn	F	CAR11018	yes	03745
2011-07-27	H228	Gronier	Mn	F	no	no	03745
2011-07-28	H584	Manta	Mn	F	no	yes	87666
2011-08-19	H707	Calanus	Mn	M	no	no	57537
2011-08-28	H755	Not named	Mn	M	AC11040	no	57537
2011-09-01	H607	Meduse	Mn	M	AC11046	yes	57537
2011-09-04	H002	Splish	Mn	F	AC11047	yes	57537
2011-09-18	H405	Barbillion	Mn	M	CAR1120	no	63607
2011-09-19	H489	Not named	Mn	Unknown	no	no	63607

Table 6. *The percentage of time whale Meduse spent gliding during ascent and descent phases of dives recorded on two occasions.*

	Meduse 0722			**Meduse 0901**
	All dives	**Dive w/ lunges**	**Dives no lunges**	**All dives**
Descent % glide	14.2 ± 14.1	13.7 ± 14.0	16.0 ± 14.6	22.5 ± 16.8
Ascent % glide	15.9 ± 12.9	18.3 ± 13.0	6.3 ± 6.5	16.9 ± 13.2
# of dives	65	52	13	17

Figure 5. *Data record of Meduse 0722. Note the relatively shallow depth of diving, with most dives <60m depth. Also, notice the spikes in speed observed throughout the record, which are indicative of lunge feeding. Yellow line indicates roll, green indicates pitch.*

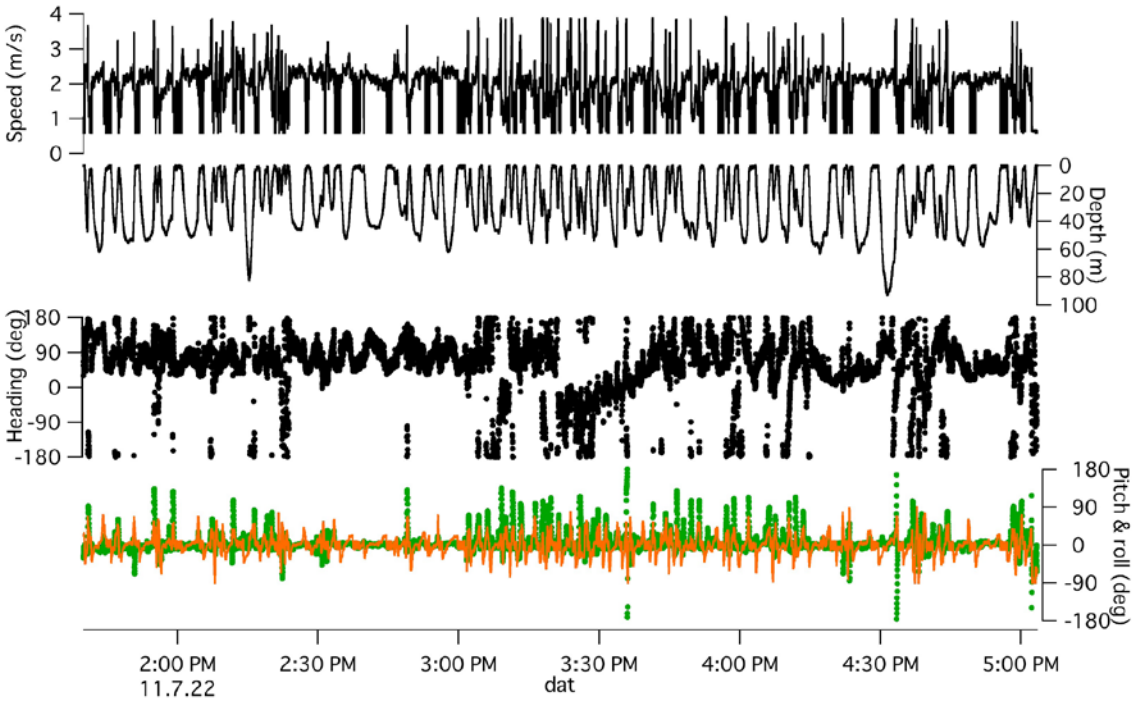


Figure 6. *Zoom-in of 15 minutes of the data record for Meduse 0722, from 3:47 to 4:02 local time. Spikes in speed correspond with changes in roll characteristic of lunging behavior.*

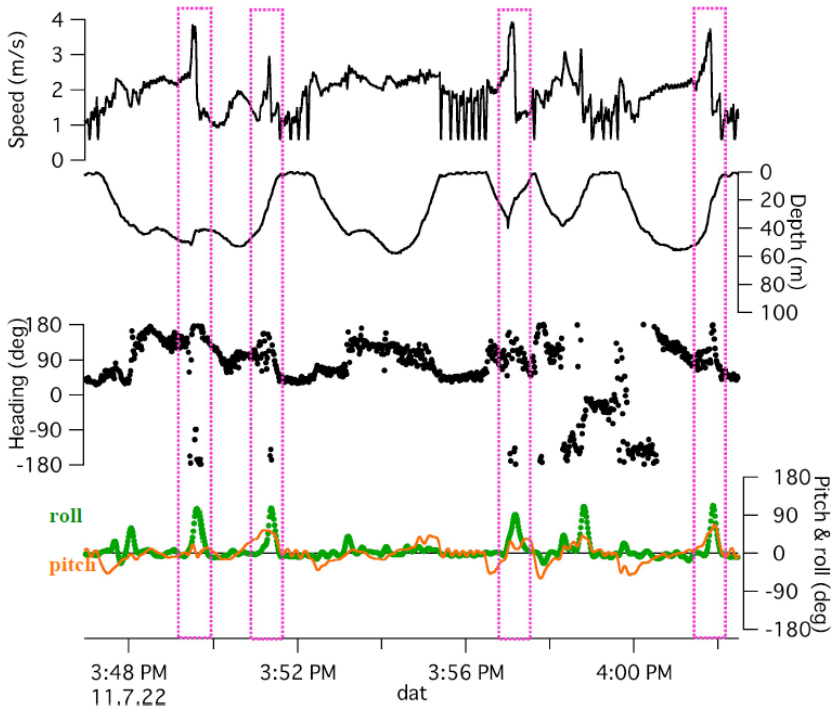


Figure 7. *Data record of Meduse 0901. In this record, there is little indication of lunging behavior, but occasional deep dives to >100m were recorded which were likely bottom feeding episodes. Such dives contain glides and appear to have good potential to apply the glide method to estimate body density. Yellow line indicates roll, green indicates pitch.*

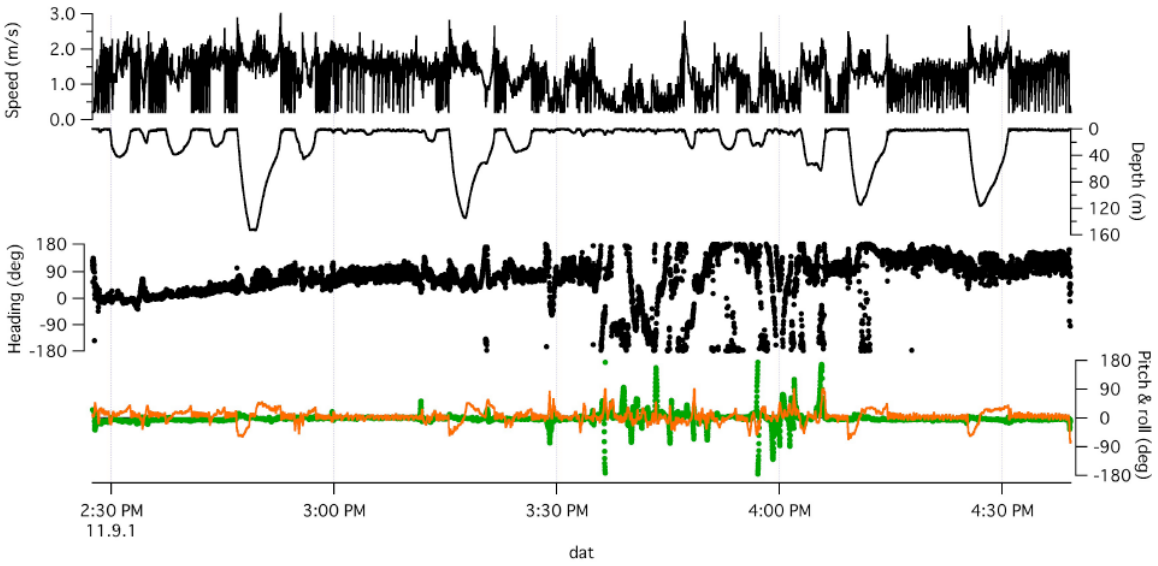
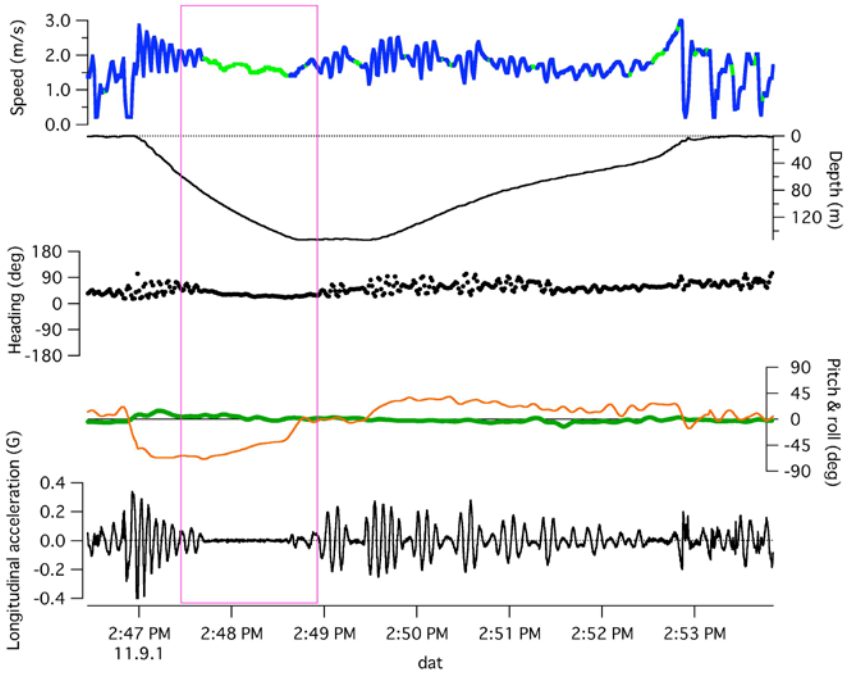


Figure 8. *Zoom-in of one deep dive from the data record for Meduse 0901. Gliding periods are shown in green in the top panel. In bottom panel, yellow line indicates roll, green indicates pitch. Note the prolonged descent glide.*



Discussion and Recommendations for Future Research

The results demonstrate that the glide method for estimating total body density can be applied to deep diving beaked whales. Our process of model-selection resulted in a very good fit between the data and the model estimates, with no apparent structure in the residuals and very high precision reflected in very narrow confidence intervals. Though the initial sample obtained here is small, the narrow range of body densities across animals gives some confidence that the precise estimates within each individual are capturing the true body density, and likely indicates that beaked whale have a narrow preferred range of body density that is maintained via allostasis.

We have had little time to fully evaluate application of the glide method to estimate the body density of humpback whales, given the full effort in analysis devoted to the bottlenose whales. For one animal that we tagged twice, early and later in the feeding season, there was no clear signal of differences in the % of time gliding during descents and ascents of dives. Inspection of a subset of the data demonstrate that humpback whales do routinely dive do depth >50 m, but that deeper dives >100 m are somewhat rare. This indicates that the effect of gases carried by the humpback whales cannot be ignored when calculating body density. For the beaked whale data, and previously for sperm whales (Miller *et al*., 2004), we were able to simultaneously solve body density and diving lung volume. That combined approach will be necessary for humpback whales.

In conclusion, we have demonstrated that the glide method for estimating total body density, and thereby body lipid stores, can be applied to certain cetacean species with low risk. Though we feel the method should be applicable to shallower divers, such as the baleen humpback whale, the more substantial effect of gases at shallower glide depths will complicate the analysis to some degree.

2) Cross-Validate the Glide-Derived Measure of Lipid Content

Analyze photogrammetry and tissue biopsy samples in order to cross-validate the glide-derived measure of lipid fat content. This section is broken into multiple subunits, each with its own evaluation and conclusion.

2.1 Laser Photogrammetry

Key Findings: Photogrammetry for humpback and Northern bottlenose whales

- Photographs were taken in the summer of 2011 in the northern Gulf of St. Lawrence (humpback whales) and in the Gully east of Nova Scotia (Northern bottlenose whales) as part of a research programs using accelerometry tags to investigate diving behavior. Two parallel lasers mounted on the camera lens were used to project dots separated by a fixed distance on the animals' body to provide a scale reference for photogrammetry. In additional photographs, the tag itself could be used to provide scale.
- The photogrammetry results suggest that this method can be used to obtain absolute dimensions of certain body parts with reasonable precision. However the method did not lend itself to taking measurements directly relevant to body condition such as girth.
- Analysis of body profiles from photographs taken from directly behind the study animal may hold potential, particularly for assessing the relative body condition of the same individuals at various point in time.
- Alternative methods of obtaining full body shape information from photographs may include aerial photogrammetry using manned or remote-controlled aircraft and stereo-photogrammetry for three-dimensional modeling.
- Underwater high-resolution sonar imaging would enable both visualization of the full body of the animal underwater, and precise measurement of distances of features from the imaging sonar. This reduces the requirement that the whale be oriented perfectly sideways to the observer position.

Background

The aim of this photogrammetry analysis was to assess the feasibility of using laser-photogrammetry to quantify the body condition of cetacean species. The target species for this pilot study were an odontocete, the Northern bottlenose whale (*Hyperoodon ampullatus*) and a mysticete, the humpback whale (*Megaptera novaeangliae*).

The photogrammetry analysis is part of a larger study to compare different means of measuring the body condition of free-ranging cetaceans. Other means of assessing lipid fat carried in the body stores included biopsy sampling, as well as an analysis of underwater gliding performance using accelerometry tags.

Specific Detailed Methods

Fieldwork on humpback whales was conducted in the northern Gulf of St. Lawrence, Quebec, Canada from 21 July to 29 September 2011. Humpback whales in this area have been subject to a long-term research project by MICS, which has provided an extensive time-line of individual life-histories, association patterns and habitat use (e.g., Doniol-Valcrose *et al.* 2007; Ramp *et al.* 2010a; Ramp *et al.* 2010b). Research on humpback whales was conducted from small (4-6 m) rigid hull inflatable boats and most animals were encountered in Jacques Cartier and Honguedo Straits close to Anticosti Island.

Photogrammetry data on Northern bottlenose whales were collected in the Gully Marine Protected Area on the Scotian Shelf east of Nova Scotia, Canada from 04 to 11 August 2011. This area is home to a closed population of approximately 160 Northern bottlenose whales (Whitehead & Wimmer 2005) Some information on ranging patterns, diet, behavior and social structure are available for these animals (Whitehead *et al.* 1997; Gowans *et al.* 2000; Dalebout *et al.* 2001; Gowans *et al.* 2001; Hooker *et al.* 2001; Hooker *et al.* 2002) making them one of the best studied populations of ziphiids to date. Research was conducted largely from a 4m inflatable boat powered by a 25hp outboard engine. A 30 m fishing vessel served as a mother ship, and some photo-ID pictures were collected from this platform.

Photo ID pictures were taken with a Canon Eos 50D digital camera with a Canon EF 70-200mm zoom lens. In addition, a Nikon D300 digital camera with a 200-4000 mm image stabilized zoom lens was used in the Gully and video recorders were employed to document the response of humpback and Northern bottlenose whales to boat approaches and tagging.

We built a custom bracket to mount two Galileo Pro-5 steerable laser pointers (Laserglow Technologies, Toronto, Canada) on the lens of the main photo ID camera (Canon Eos 50D). This device projected two green dots 100mm apart onto the flank of the photographed whale. The design was modeled on that of Durban & Parsons (2006). The distance between the dots was calibrated at the start and the end of each research cruise and no detectable deviation from 100mm was noted. Distances in the photographs were measured (in pixels) using Adobe Photoshop Elements 6 for the Mac (Adobe Systems Inc., San Jose, USA) and converted to absolute distances by using the laser dots or, for tagged individuals, the length of the housing of the accelerometry tag (see below) for scale. To determine the distance over which the laser dots could be detected we noted for each photograph whether the laser device was turned on or not. During the fieldwork in the Gulf of St. Lawrence, his information was noted using the Logger software (International Fund for Animal Welfare, Yarmouth, USA). In the Gully, the information was taken using paper field notes.

The biologging component of the study used Little Leonardo 3MPD3GT data loggers. These can be attached to the flank or back of cetaceans with a single suction cup and are deployed using a carbon-fiber pole. The data logger itself had a diameter of 26mm and a length of 175mm and was housed in a buoyant housing with a length of 215mm. A small section of the black data logger protruded from the housing, but for calibration purposes only the white housing, which contrasted well with the dark flanks of the animals, was used.

Results and Outcomes

1) Performance of the laser photogrammetry device

We collected 2460 photographs of humpback whales and over 1800 identification photographs of Northern bottlenose whales. For photographs that the field notes stated were taken with the laser-photogrammetry device turned on, the laser dots were not detectable in about half of these (Figure 9). Even though range measurements were not taken in our study, it seems that the laser dots are increasingly difficult to detect at distances exceeding 15m. At these distances the only a small part of the body was visible within the frame of our camera set-up thus precluding the measurement of large body sections such as the distance between dorsal fin and blowhole. Detectability is also highly dependent on ambient light conditions with overcast and back-lighting conditions greatly improving the visibility of the lasers (see Figure 10).

Figure 9. *Photographs of the dorsal fin of a humpback whale (left) and a Northern bottlenose whale (right) showing the dots projected by the laser-photogrammetry device. The arrows point to the left of the two dots and the distance between the dots is 100mm.*

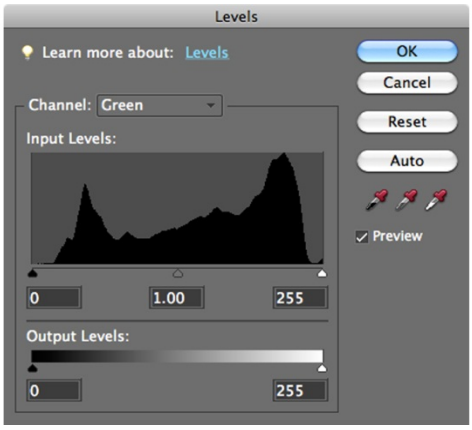
Presumably because of their lighter body coloration, the laser dots were generally better visible on Northern bottlenose whales compared to the near-black humpbacks (Figure 9). In a few instances where humpbacks performed pectoral slaps close to the boat, we were able to obtain photographs showing the laser dots on white parts of the pectoral fins (Figure 10). These were much more visible when the fin was back-lit.

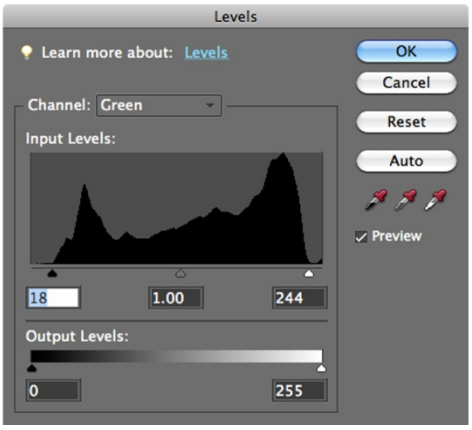
Figure 10. *Photograph showing the two dots from the laser photogrammetry device on the pectoral fins of humpback whales in a back-lighting (left) and a front-lighting (right) situation. The photographs were taken roughly at the same distance. Arrows point to the left/top laser dot. Note that the right-hand photograph was taken in portrait format.*

2) Enhancing the visibility of lasers

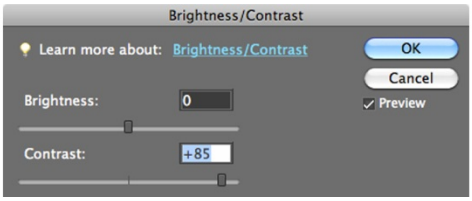
The goal of this analysis was to test whether modifying the color balance, contrast and saturation of the photographs would improve the visibility of the laser dots, thus allowing them to be detected in photographs taken at greater ranges and therefore showing a greater proportion of the animal's body (Figure 11). The modifications were done using the 'Adjust Lighting' feature in Adobe Photoshop Elements 6 for the Mac. Several approaches were tried and the following consistently provided increased detectability of the laser dots:

- Optimize the levels of the green channel by setting the upper and lower limits at the edges of the color curve:





- Increase the contrast by 80-90% and adjust the brightness to maximize visibility of the dots:



These adjustments resulted in an improved detectability of the laser dots, while still maintaining the visibility of the outline of the animal's body and many of its natural markings for measurement purposes.

Figure 11. *Photographs of the dorsal fin of a humpback whale showing the laser dots before (left) and after (right) digital enhancement. The arrows point to the left of the two dots and the distance between the dots is 100mm.*

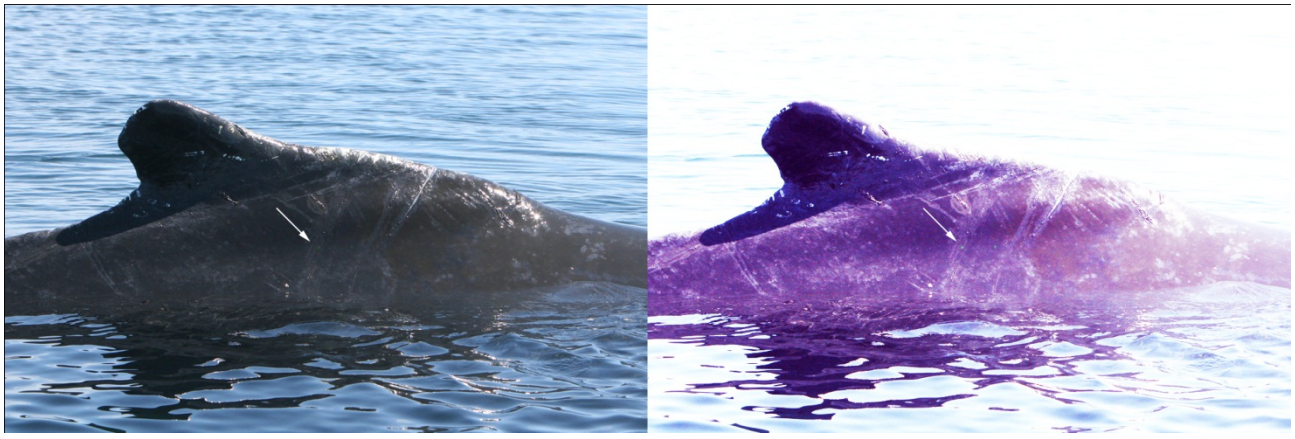


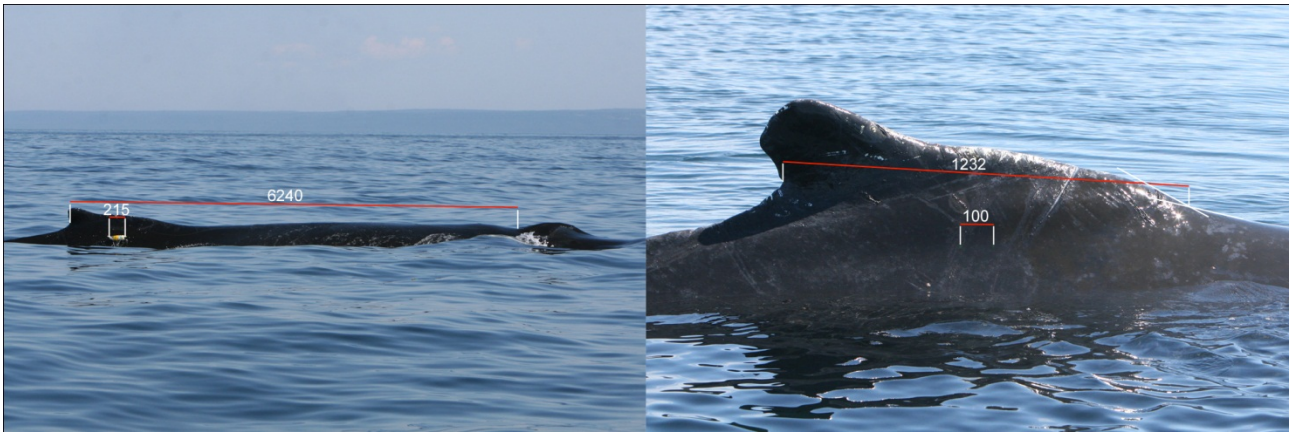
Figure 11 shows a comparison between an unmodified and a digitally enhanced photograph. Adjusting levels and increasing contrast and brightness allowed the detection of the laser dots in a few photographs where they had been missed previously. However even with this improvement it was still only possible to detect the laser dots in photographs taken at relatively close range.

3) Photogrammetry of humpback whales

Three kinds of animal measurements could be obtained from the photographs: 1) Distance from blowhole to the dorsal fin, 2) length of the dorsal hump and 3) length of the pectoral flipper. The distance from blowhole to dorsal fin was defined as the length of a straight line drawn from the lowest point of the depression behind the blowhole to the deepest point of the notch behind the dorsal fin (see Figure 12, left). Length of the dorsal hump was defined as the straight-line distance between the deepest part of the depression at the front of the dorsal hump and the deepest point of the notch behind the dorsal fin (see Figure 12, right). These features were chosen because they could be consistently identified in good photographs and were comparatively little affected by individual variation (e.g., dorsal fin shape). However the anterior end of the dorsal hump was poorly defined in some individuals. This feature was therefore also referenced to natural markings on the animal to make measurements as consistent as possible. At the close ranges necessary to detect the laser dots it is impossible to take photographs showing both blowhole and dorsal fin and all these measurements were referenced to tags. Measurements of dorsal hump length were referenced to either tags or laser dots and in a few cases to both. In two instances where laser dots were visible on pectoral fins (photos IMG_0264 and IMG_1156/57), the distance between the middle of the fin at the insertion to the body and the fin tip was measured and referenced to the laser dots. Body length, or any body feature allometrically related to body length, is an important parameter to describe body condition, as body shape parameters should be scaled to overall body length.

All measurements taken from photographs are listed in Appendix I. Blowhole to dorsal fin distance could be measured in four photographs, two from individual *Meduse* (H604), and one each from individuals *Manta* (H584) and H755. Estimates of this distance ranged from 5400mm (H755) to 6240mm (H584) and the two measurements taken for H604 during the same sequence of surfacings differed by approximately 2%. Measurements for this individual as well as for H755 were derived from slightly angled photographs (see Appendix I).

Figure 12. *Photographs showing the measurement taken for humpback whale photogrammetry. Long red lines give blowhole to dorsal fin distance (left) and dorsal hump length (right). Short red lines show the reference distance (length of the tag in the left photo and distance between the laser dots in the right photo). All distances are in mm.*



Measurements for the length of dorsal hump ranged between 690mm and 1686mm with an average length of 1228mm. For this parameter, measurements taken for the same individual

on different occasions differed by 1-33% with an average difference of 12%. The comparatively great individual difference, as well as the large measurement error may be partially due to the fact that the shape of the dorsal hump showed great individual variation and that the anterior edge of the hump was not very clearly defined for some individuals. It is difficult to assess how much of the variation in dorsal hump is due to differences in nutritional condition, however, since shape of the dorsal fin and hump are highly stable over time (Blackmer *et al.*, 2000), it is likely that most of the variation is individual rather than caused by differences in body condition.

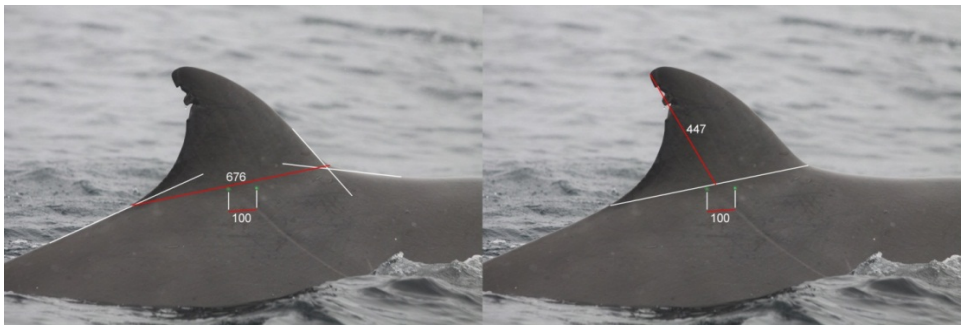
The length of the pectoral fin was measured for three unidentified individuals and ranged from 2422 to 2873mm with an average of 2683mm. No repeat measurements of this parameter could be obtained making it impossible determine the precision of this measurement.

While none of the measurements are immediately indicative of lipid-content body condition, they may prove useful in the future for establishing body size as well as for identifying reference points on the animal's body for body condition measurements. However, to achieve this, efforts must be made to reduce the measurement error (e.g., by establishing stricter criteria with respect of the orientation of the animal to the camera, aiming for tag placements on parts of the flanks that are reasonably parallel to the animal's body axis, and incorporating markings indicating orientation and scale on the tag itself; see below).

4) Photogrammetry of Northern bottlenose whales

The tag was only clearly visible in a single photograph of Northern bottlenose whales (IMG_6630.jpg), so that most measurements were referenced against the laser dots. Because laser dots were never visible at ranges where both the blowhole and dorsal fin were photographed in the same frame, the analysis was restricted to measurements of the dorsal fin. Width of the dorsal fin was defined as the straight-line width of the base of the dorsal fin. The anterior point of this distance was defined as the point of intersect between two lines drawn along the back of the animal anterior to the fin and the leading edge of the dorsal fin (see also Durban & Parsons 2006). The posterior point was defined as the point where the curve of the fin deviated from a straight line drawn along the upper part of the caudal peduncle (Figure 13). The height of the dorsal fin was defined as the distance between the fin tip and the mid-point of the line used to measure fin width. In addition, the ratio of height to width of the dorsal fin was also analyzed, as it might provide information on age and/or sexual dimorphism (see e.g., Heimlich-Boran 1986).

***Figure 13**. Photographs showing the measurement taken for photogrammetry of Northern bottlenose whales (Individual I). Long red lines give basal width of the dorsal fin (left) and height of the fin (right). Short red lines show the distance between the laser dots. All distances are in mm.*



Photogrammetry data for Northern bottlenose whales are summarized in Table 7. Measurements for dorsal fin width and height could be obtained for 9 different individuals, and for five of these repeat measurements were available. The width of the dorsal fin at its base ranged from 431mm (Individual H) to 676mm (Individual I), and dorsal fin height ranged from 392mm (Individual D) to 456mm (Individual C). The height to width ratio of the fin ranged from 0.769 (Individual A) to 1.034 (Individual H) with no clear bimodal distribution indicative of sexual dimorphism.

***Table 7.** Summary of the photogrammetry data collected from nine Northern bottlenose whales. Measurements are given as mean and standard deviation (St. dev.)*

Individual	**# of photographs analyzed**	**Width of fin base (*mm*)**		**Fin height (*mm*)**		**Height/Width Ratio**	
		Mean	***Stdev.***	***Mean***	***Stdev.***	***Mean***	***Stdev.***
A	9	582	48	447	34	0.769	0.028
B	8	479	22	416	20	0.870	0.024
C*	4	507	66	456	34	0.906	0.061
D	2	444	20	392	1	0.885	0.042
E	1	570	-	444	-	0.779	-
F	4	465	33	403	15	0.871	0.086
G	1	511	-	423	-	0.826	-
H*	1	431	-	446	-	1.034	-
I	1	676	-	447	-	0.662	-

** tagged individuals*

Repeat measurements of the same individuals were found to be reasonably consistent with measurements typically differing by between 0.2 and 13%. Dorsal fin height-width ratio, a relative measure, tended to have smaller relative errors associated with them. This may

suggest that the measurement of the spacing of the laser dots or the plane on which they were projected (i.e., on the dorsal fin itself or on the flank below the fin) were major sources of error. This error may be further reduced by setting more stringent criteria as to the orientation of the animal with respect to the camera.

Again, none of the parameters measured provided direct information on body condition. However, with greater sample size and concurrent genetic sexing of individuals, the height-to-width ration may ultimately provide a non-invasive means of distinguishing mature males and females in the field and could therefore prove valuable for future studies on the social structure, life-history and sex-specific variation in body condition of this species.

Discussion and Recommendations for Future Research

To date, only aerial photogrammetry has provided useful information to detect seasonal variation in the body condition of free-living cetaceans (Perryman & Lynn 2002). The downside is that photogrammetry from commercial aircraft is very costly, precluding its application to a wide range of cetacean studies particularly for offshore species like beaked whales. While aerial photogrammetry has the potential to deliver concrete answers to questions about nutritional status and body condition, there are alternative methods that may be worth exploring, although these are currently still associated with greater risk of failure and uncertainty. In addition to exploring other methodologies for assessing body condition, the system used in the current study can be improved further to supply reliable measurements of body size. Alternative approaches include use of stereo-photogrammetry to measure body dimensions, possibly using underwater imaging sonars, or to use details of body shape to estimate body condition, similar to the methods of Bradford *et al*. (2012). Finally, an alternative approach using an underwater imaging sonar is introduced.

Improving the current system:

While the laser-photogrammetry system used will probably not provide direct information about body condition on its own, it gave valuable measurements of body size that can complement other measurements in order to assess body condition of free-ranging cetaceans. Testing the system on two different cetacean species in the two field sites helped identify the following modifications of the apparatus and technique to improve its effectiveness:

1) Improve visibility of the lasers: One limitation of the system was that the laser dots used for reference could only be detected at short ranges (typically less than 15m). Increasing this range would increase the proportion of the animal's body that can be photographed (particularly for large species such as humpbacks) and increase the number of useful photographs that could be obtained in a given encounters. The two laser pointers used in the current study consistently differed in their detectability with the left laser being less intense and more diffuse than the right one. This was a specific problem with our unit, and replacing the left laser with a better unit would lead to a slight improvement in detectability. In addition it may be worthwhile to see if other colors (e.g., red) are more detectable, especially on the dark skin of humpback whales. A final consideration would be to switch to a more powerful laser, however, this creates risks and safety hazards for the field personnel.

2) Set stricter criteria for photograph selection: Given the limited number of data available for this analysis, all photos that showed the animal roughly perpendicular to the camera and included a scale reference were used. However, maximizing the photographic database came with the cost if increased measurement errors. Limiting photographs strictly to those with the animal at an orientation of 90º or 270º with respect to the camera would help decrease the

parallax error generated when the whale is not parallel to the plane of the camera (Durban & Parsons 2006). However, determining the orientation of the animal in the frame is often difficult and potentially subjective. Another source of error arises from situations where the laser dots are at a different distance from the camera (e.g., low on the flank of the animal) compared to the distance measured (e.g., the dorsal fin). Setting criteria of only selecting photographs where the measurements and scale reference are at the same focal plane (e.g., laser dots on the dorsal fin for dorsal fin measurements) will reduce this type of error. However, especially for large bulky cetaceans such as humpback whales this will be difficult to achieve.

3) Use different lenses to maximize the proportion of the body photographed: In this study we used a camera set-up that was designed for taking high-quality photographs of cetaceans for identification purposes. However such a set-up may not be ideal when the main aim is to take photographs for photogrammetry measurements. In the current study it would have been desirable to obtain pictures showing a large proportion of the animal's body to obtain precise estimates of overall body length (e.g., in the form of dorsal fin to blowhole distance). Switching to lenses with a shorter focal length (e.g., 50-100mm), especially when animals are close to the boat may be desirable here.

4) Use different camera settings and photographic techniques to improve laser detectability: Much like the telephoto lenses, the technique and camera settings we used in the current study were optimized for photographic identification of individuals, but may not have been ideal for photogrammetry. Whereas backlighting is to be avoided when taking identification photographs, as it makes the detection of natural markings difficult, it was found to greatly improve the visibility of the laser dots (see Figure 9). Similarly, changing the camera settings from those typically used for photo ID may be useful. Adjusting the exposure settings and lowering the ISO settings, especially on bright days, may be particularly effective.

Alternative approaches

1) Aerial photogrammetry using remote-controlled aircraft. Remotely controlled unmanned helicopters are available in a variety of cost-ranges, and many systems are capable of carrying photographic or video equipment. Wildlife film crews are already relying to a great extent on remote-controlled aircraft to obtain footage of animals with a minimum of disturbance and future improvements in technology and reduction in costs are only likely to make such approaches more attractive. The fact that aerial photogrammetry requires photographs to be taken from straight above the animal (Perryman & Lynn 2002; Mocklin *et al.* 2010) poses technological challenges, but gimballed systems shooting several frames a second or recording continuously using high-definition video may be able to provide useful data. However, any system will likely only ever be useable in good conditions and at wind speeds of less than 10 knots, limiting their applicability in the more exposed parts of the world's oceans.

2) Boat-based stereo-photogrammetry. Earlier research using stereo-photogrammetry to measure body size of cetaceans (e.g., Dawson *et al.* 1995; Bräger & Chong 1999; Bräger *et al.* 1999; Chong & Schneider 2001) was largely abandoned in favor of calibrated photography using laser range-finders or laser-photogrammetry systems. Stereo-photogrammetry requires the installation of bulky camera mounts on the research vessel. It is only effective at short distances and thus requires extremely close approaches to the study animals. However, stereo-photogrammetry has the advantage that it allows (albeit limited) measurement in three-dimensions potentially making it useful technique to obtain

information related to body condition. Photographic modeling software has undergone rapid improvement since the times when stereo-photogrammetry was last used on free-ranging cetaceans (e.g., de Bruyn *et al.* 2009) and analyzing body condition using three-dimensional models of the animals' body based on stereo-photographs may provide a viable route. An alternative form of this approach is to use underwater imaging sonars (i.e., BlueView P900-130) to conduct stereo photogrammetry underwater.

3) Morphological assessment of body condition. While it may be challenging to assess body condition in a quantitative fashion, many cetaceans show signs of nutritional stress that can be identified visually, at least in extreme cases. For example, grey whales show characteristic signs of emaciation in the form of a depression behind the blowhole, as well as visible vertebrae, shoulder blades and ribs following summers with high and persistent ice cover limiting access to the Bering Sea feeding grounds (Le Boeuf *et al.* 2000; Bradford *et al*., 2012). In right whales (*Eubalaena spp.*) blubber thickness measured using ultrasound is related to reproductive status and food abundance (Miller *et al.* 2011). Similarly, data for minke whales (*Balaenoptera acutorostrata* and *B. bonaerensis.*) suggest that blubber thickness along is a good indicator of total blubber mass, and is correlated with food abundance (Naess *et al.* 1998; Konishi 2006). Lactating blue whales (*Balaenoptera musculus*) in the Gulf of California typically show signs of nutritional stress in the form of the vertebrae being visible along the dorsal ridge in front of the dorsal fin (Christian Ramp, *pers. comm.*).

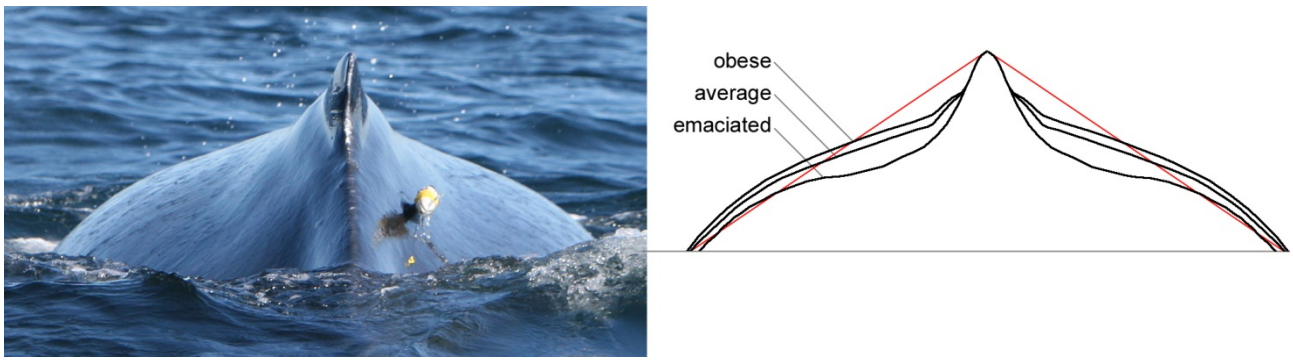
Looking for similar signs of nutritional stress in the species investigated by the current study may prove to be a viable route towards assessing body condition, at least in cases of severe nutritional stress. Such an index would be primarily qualitative (e.g., scoring the visibility of vertebrae from photographs taken under standardized conditions), but quantitative methods may be feasible as well. The method of Miller *et al.* (2011), which allows the measurement of blubber thickness in free-ranging cetaceans using an ultrasound transducer mounted on the end of a cantilevered 12m carbon-fiber pole mounted on the bow of a small vessel, may prove effective for humpback whales and Northern bottlenose whales as well. Especially for humpbacks, a quantitative analysis of photographs taken from directly behind the animal may prove effective.

Figure 14 shows a comparison of this aspect between a severely emaciated animal and one presumably in average nutritional state (Individual *Barbillion*, H405). This comparison clearly shows the loss of tissue mass along the sides of the spinal column and the dorsal hump, presumably due to loss of both blubber thickness and muscle mass. While the differences are obvious in such extreme cases, more subtle changes in body condition may be detectably by a quantitative analysis of the curvature of the body from standardized photographs taken from behind the animal.

Figure 14. *Photographs showing the posterior view of humpbacks severely emaciated (left) average (right) body condition. Left photograph by Catherine Dubé - MICS photo.*

Such differences in body curvature could be quantified by measuring the proportion of the body trace lying above and below a reference line drawn from the ridge of the dorsal fin to the point where the body trace meets the water (Figure 15). This analysis would be very sensitive to the point in the surfacing sequence at which the photograph is taken, as the cross-section of the body changes along the main body axis. However, this could be standardized by taking continuous photographs from directly behind the animal and selecting the picture where the tip of the dorsal fin forms the highest point of the body contour. The analysis is also sensitive to the height of the surfacing, the proportion of the body that is visible above the water. This is particularly problematic, as this proportion may be directly influenced by the animal's body condition due to differences in buoyancy. The height of surfacing may be standardized to some degree by choosing only pictures taken before the animal fluked up. However, animals in extremely poor body conditions may only show their flukes rarely. Even though the comparison of body condition between different individuals may be difficult using this method, it holds great potential for assessing the relative body condition of the same individuals at different points of time, as variation in the height of surfacing can be accounted for by aligning photographs digitally using natural markings on the dorsal fin and tailstock.

Figure 15. *Photograph and actual tracing (line labeled 'average') of the posterior aspect of a humpback whale. Hypothetical tracings of the same animal in obese and emaciated body condition are also shown. A reference line from the ridge of the dorsal fin to the water line is shown in red.*



4) High-resolution underwater imaging sonar. Photographic approaches to measure body shape make use of our ability to see the whale above the surface of the water. Two factors

have dominated our evaluation of laser-photogrammetry to study body condition: inability to observe the full body of the animal, and errors caused by the orientation of the whale body not oriented sideways to the camera.

A novel approach which could potentially overcome both of these obstacles would be to use high-resolution underwater scanning sonars to image the body of the whale while it is underwater. Typical scanning sonars operate at frequencies of 450-900 kHz, which provides a measurement resolution of 1-2cm. Scanning sonars have been widely used to scan and measure underwater features (see http://www.opentheoceans.com/sonarvideos.htm; Zerr & Stage, 1996). When whale subjects are closely approached for tagging, they could be tracked underwater using scanning sonar, and the sonar returns later could be used to reconstruct the body shape and dimensions of the whale.

2.2 Blubber Lipid Quantification

Key Findings: Body condition estimation from total blubber lipid content in cetaceans

- Previous research has shown that nutritive condition in cetaceans can be estimated from the total lipid content (% lipid) of the blubber. However, stratification and layering of the blubber lipids appears to vary by species. If total lipid estimates are based on shallow, remote biopsy samples, knowledge of the species-specific relationships between the proportions of lipid in the superficial layer to those in the blubber layer as a whole is required. Indeed, if this relationship is not consistent then full thickness samples are needed for this method to be sufficiently robust.
- The proportion of lipid in the blubber is not linearly related to blubber thickness. A thinner layer of blubber may contain proportionally as much lipid as a thicker layer. Data from stranded and by-caught harbor porpoise and harbor seals suggests that the relationship may be non-linear, with an exponential increase to an asymptote. Thus, below this asymptotic threshold, animals with thinner blubber layers have lower lipid levels which are indicative of poor nutritive condition. However, this relationship is likely also species specific, depending on the other functions of the blubber such as thermoregulation and hydrodynamics.
- Considerable variation was seen in the blubber lipid content across the bodies and through the blubber layer of the individuals sampled in these various sub-projects. The relationship between lipid content and blubber depth appears to be consistent across species and highlights the inadequacy of superficial biopsy samples to estimate blubber lipid content, and thus by extension, body condition. However, the distributional pattern of blubber lipid content likely differs across species and definitely between taxonomic groups, and further investigation is required to understand these patterns of distribution.
- This having been said, the variation in the proportion of lipid, if obtained using full thickness biopsy samples collected consistently from the *same* site of the *same* species, may be used to infer differences in nutritive condition between individuals.
- Key research questions for the future are whether there is a consistent relationship between the lipid content obtained from these biopsy samples with body condition, and whether this relationship can then be used to validate the glide models requires further investigation.

Sub-project 1: Variation in Odontocete Blubber Lipid Content

Background

The aim of this study was to determine whether the total lipid content of blubber samples collected by remote biopsy from cetaceans could be used as a surrogate for body condition (Aguilar & Borrell 1990), and whether this method could therefore ultimately be used to cross-validate the glide models. The way in which lipids are distributed through the blubber depth and across the body of cetacean species will affect the lipid content of blubber samples collected by remote dart biopsy. In order to determine variation in total lipid by these two factors, a study using samples from freshly dead odontocetes, stranded around the UK coast, was carried out. The main aim of this research effort was to investigate how reliable body condition estimation using total blubber lipid content from a single biopsy sample taken around the dorsal region would be in light of the potential variation by depth and body location.

Specific Detailed Methods

Five individuals of different cetacean species of various sizes were sampled; sperm whale (*Physeter macrocephalus*), long finned pilot whale (*Globicephala melas*), bottlenose dolphin (*Tursiops truncatus*), striped dolphin (*Stenella coeruleoalba*) and harbor porpoise (*Phocoena phocoena*). Between 2.5cm^2 and 5cm^2 blocks of full depth blubber were taken from different sites across the body of each individual; dorsal to ventral (top to bottom) and anterior to posterior (rostrum to tail). Between 12 and 18 samples were collected from each animal except the sperm whale, which provided only three samples. The thickness of the blubber layer was measured to the nearest millimeter. Cetacean blubber is not a homogenous tissue with a uniform distribution of lipids, but is in fact stratified into horizontal layers from the skin down to the muscle which can be differentiated visually, histologically and biochemically in many species (Aguilar and Borrell, 1990). In most cases, three distinct layers have been identified, and it has been suggested that each blubber layer, with a different lipid content and composition, has a particular function. For this reason, blubber samples were divided horizontally into either three or five layers of equal size depending on the species, thickness of the blubber, and presence of visually distinctive characteristics. Duplicate subsamples of 0.25g each were taken from the outer layer, closest to the skin, the middle layer, and finally the inner layer, closest to the muscle. The total lipid was independently extracted from these subsamples using a modified version of the Folch *et al*. method (1957).

Blubber samples were homogenized to release the lipids with a 2:1 dichloromethane-methanol mixture containing 0.05% butylhydroxytoluene (BHT) with a 1:21 ratio of tissue (g) to solvent (ml). The homogenate was then mixed thoroughly with 0.25 x its volume of 0.9% potassium chloride (KCl) solution using a mechanical shaker for 10 minutes. The mixture was then centrifuged at 900 relative centrifugal force (*rcf*) for 20 minutes to separate the solution into two immiscible phases. The upper phase was removed and discarded and the lower phase rinsed with four times the original mass of the blubber sample in anhydrous sodium sulphate (Na_2SO_4). After rinsing the lipid extract was transferred to a pre-weighed tube and the solvent evaporated under air at 30^{o}C for four and a half hours. The tube was then left at room temperature to complete the evaporation overnight. The tube was finally re-weighed to determine the mass of total lipid extracted and the lipid content expressed as a percentage of the wet weight of the sample. Statistical analyses were carried out using the program R, version 2.11.1 (R Core Development Team 2011), using analysis of variance

(ANOVA) to compare mean blubber thickness by body location (dorsal to ventral and anterior to posterior), and quasi-binomial generalized linear models with a logit link function to determine total lipid content variation by blubber thickness, blubber layer (outer, middle or inner) and body location. All analyses were carried out in duplicate.

Results and Outcomes

Blubber thickness varied by body location in two out of the five individuals (the pilot whale and bottlenose dolphin). However, the generalized linear models revealed that lipid content was not correlated with blubber thickness for any of the five individuals (all p values >0.05).

Lipid content did vary by body location, both between and within individuals. The lowest was 34.1% ± 0.94% from the sperm whale ranging up to 92.1% ± 3.53% in the harbor porpoise. The variation was much less (~10%) in the animal with highest blubber lipid levels than the animal with the lowest (~35%). In general the levels were highest in the ventral or lateral samples whereas vertical body location from anterior to posterior was not significant in the models, except when included as an interaction term with either one or both of the other body location variables.

In order to investigate the difference between the 'target' area where live biopsy samples would be obtained (i.e., mid dorsal region) and the dorsal area from rostrum to tail, or the rest of the body as a whole, paired t-tests were carried out to determine if there were significant differences in the average lipid content of the samples from these regions. In two animals (the bottlenose dolphin and striped dolphin) the target area had a statistically significant lower lipid content that the rest of the body ($p<0.05$). However, in the two other individuals (excluding the sperm whale for which there were insufficient samples) there was no difference between the target area and either the dorsal region or the rest of the body. Only in the bottlenose dolphin was the target area significantly different to either the dorsal region or the rest of the body. The results are therefore somewhat equivocal and a much larger sample from animals of the same species is required to investigate this fully.

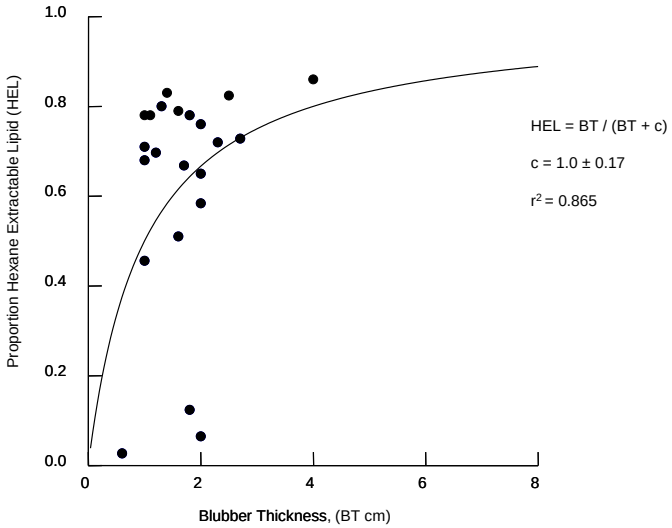
Of more importance were the layer and depth of the sample indicating significant stratification of lipid content within the blubber. Except for the harbor porpoise, whose blubber lipid levels were extremely high across all sites and subsamples (all > 82%), the middle layer had consistently the highest lipid levels among all individuals. Thus, sample depth does appear to be an important factor in ensuring that the body condition estimate obtained from blubber lipid content is unbiased and reliable.

Discussion and Recommendations for Future Research

In all the species studied, blubber thickness was not related to blubber lipid content, a result which has also been reported by other studies (Read 1990, Evans *et al*. 2003, Koopman 2007) suggesting that blubber thickness is a relatively poor index of nutritive condition. Data from stranded and by-caught harbor porpoise (Jepson, P. personal communication) and a limited sample of harbor seals (Hall *et al.* 1999), suggest the relationship may in fact be non-linear, asymptotic exponential (see Figure 16). Thus, below a certain threshold the relationship between thickness and lipid content may be linear. Above this threshold as the relationship reaches an asymptote, higher lipid levels are not related to thickness, where a thinner blubber layer may contain proportionally as much lipid as a thicker layer. Hence animals with levels below a particular lipid content threshold are very likely to be in poor condition. Where this threshold occurs may differ between species. In addition, species specific differences in the extent to which blubber can be mobilized are limited by additional thermoregulatory and

hydrodynamic considerations apart from the function of blubber as an energy store (Koopman 1994). Quantifying blubber lipid content rather than measuring blubber thickness may therefore provide a more robust estimation of an individual's body condition.

Figure 16*. The relationship between blubber thickness and lipid proportion in harbor seals was significant at the 10% level (p = 0.0939). Figure reproduced from (Hall et al. 1999). A similar relationship was also found among stranded and by-caught harbor porpoise (n=358).*



Blubber lipid content varied significantly by horizontal body location (dorsal to ventral) except for in the individual in excellent body condition with very high lipid levels (>80%) in the blubber. Animals in sub-optimal condition had higher levels in the ventral region than the dorsal. These results were similar to those found in harbor porpoise and common dolphin (*Delphinus delphis*) where the highest lipid levels were found in the anterior-ventral region (Koopman *et al*. 2002, Tornero *et al.* 2004). Vertical body location (anterior to posterior) showed much lower or insignificant variation.

Comparisons of the lipid content between the remote biopsy sampling target area to the average blubber lipid content in the rest of the animal among four of the individuals revealed differing results. For two animals the differences were not significant but for the two others the differences were significant. This may therefore suggest that the results from biopsy samples collected from this area may not represent the body condition of individual odontocetes.

However, in fin (*Balaenoptera physalus*) and sei (*Balaenoptera borealis*) whales, two species more closely related to one of the study species, the humpback whale, the dorsal mid and posterior areas (the biopsy target area for mysticete species) were shown to be the major sites of lipid storage, and unlike the smaller cetaceans in our study, the anterior and ventral blubber contained the least lipid (Lockyer *et al.* 1985). In a sample of 20 fin whales collected by Icelandic whalers in late July-mid August, the mean lipid content (%) of samples from the *mid* dorsal region was 56.2% ± 1.1% compared to between 29.1% ± 1.1% to 53.2% ± 3.4% from elsewhere. However, among 12 sei whales also sampled, the *posterior* dorsal had the highest lipid content with 70.5% ± 1.2% compared to between 39.2% ± 4.1% and 67.7% ± 2.1% for the rest of the body. These findings in addition to the results of our smaller study demonstrate the variability both among and between species of the same suborder and

between individuals. These differences are probably due to the combination of the different hydrodynamic body shapes and maneuverability of the species as well as their life histories that determine their utilization of their endogenous energy stores.

The distributional patterns of blubber lipids therefore appear to differ broadly across taxonomic groups, so biopsy sampling from the same site on different species may produce differing results. There have only been a few detailed studies into the variation among fat stores across the body probably due to the limitations of sample availability. However, based on those that have been conducted on mysticetes (Lockyer *et al.* 1985, Aguilar & Borrell 1990), the standard procedure of live biopsy sampling from the mid/posterior dorsal region may provide a sufficiently representative sample for body condition inference and can thus be used as a cross-validation for the dive models. With regards to the smaller odonotocete species, given the variability in blubber lipid content across the body seen in this small study, additional species-specific studies are required to establish whether dorsal remote dart biopsies are representative of the lipid stores of the individual as a whole. However, relative differences in body condition between individuals may be inferred if the samples are taken consistently and reliably from the same site on the body from individuals of the same species. Potential individual variability can therefore be assessed and this information can be used to compare with the glide models.

In terms of sex related differences in blubber lipid content, none were seen in this small study. However, Aguilar and Borrell (1990) found significant differences in the blubber lipid content of fin whales of different reproductive categories. Pregnant females were seen to have the highest blubber lipid content and lactating females the lowest, with males and immature animals in between. There were also differences between the blubber lipid content of different reproductive classes in striped dolphins (Gomez-Campos *et al.* 2011), but these were not consistent with the differences seen in the fin whales mentioned above. However, Evans *et al*. (2003) found no significant relationship between blubber lipid content and the total length, age or sex of sperm whales. There is no information on how sex may affect the topographical distribution on of blubber lipids across the body of individuals however. As such, any variation in blubber lipid content with respect to sex and age requires further investigation and may also demonstrate species specific differences.

Finally, in terms of the results of the stratification of the blubber layer, the evidence that the middle layer of blubber had higher lipid content than either the inner or outer layers has also been confirmed by others in baleen whales such as fin and sei whales (Lockyer *et al.* 1985) and in odontocetes such as sperm whales (Evans *et al.* 2003) bottlenose dolphins (Shoda *et al*. 1993), killer whales and belugas (Krahn *et al*. 2004). Results from this investigation and other studies therefore suggest that, regardless of body location, attempts should be made to sample the middle blubber layer when estimating body condition using remote biopsy sampling. Therefore, to obtain representative blubber lipid results to cross-validate the glide model with most confidence, biopsy samples should penetrate down to at least the middle blubber layer of the individual. Ideally the entire blubber layer should be sampled to ensure a more complete estimate, but obviously this may not be practical or logistically possible for some species.

Any biopsy sampling scheme, after accounting for the potential species-specific differences already discussed, needs to be consistently applied in order to derive any confidence in the blubber lipid content–body condition relationship and its subsequent use in the cross-validation of the glide models. Integrated approaches using both morphological body density estimates in addition to the blubber lipid measurements in biopsies taken from an appropriate

location and to a sufficient depth would provide the best estimate of size of an animal's energy stores.

Sub-project 2: Total Blubber Lipid in Northern Bottlenose Whale Samples

Specified Detailed Methods

Blubber samples collected from a Northern bottlenose whale that stranded in Bunnefjorden, Norway were analyzed for total lipid content. The animal was a female in good condition with a blubber layer ranging from 55mm to 65mm in thickness. Six biopsy samples were then collected from this individual using the ARTS biopsy system from 10 meters using three different Larsen biopsy tips (20mm, 40mm, and 60mm as shown in Table 8). The sample area was just below the dorsal fin on the left side. The blubber thickness in this area was found to be 56mm-59mm. The samples were divided in two, vertically to provide duplicate samples for hormone analysis as detailed below.

Results and Outcomes

The results are shown in Table 8.

Table 8. *Total lipid in Northern bottlenose whale biopsy samples.*

Sample		Biopsy Tip (mm)	Sample Depth (mm)	Thickness Measured in Laboratory (mm)	Blubber Mass (g)	Lipid (%)
1	Whole Sample	20	12	10	0.0914	**52.63**
2	Whole Sample	20	13	11	0.1181	**43.18**
3	Whole Sample	40	30	30	0.3655	**60.14**
4	Whole Sample	40	10	8	0.0807	**65.80**
5	Whole Sample	60	51	53	0.1832	**68.50**
6	*Outer Layer*				0.1213	58.53
6	*Middle Layer*				0.095	75.58
6	*Inner Layer*				0.0985	76.55
6	Whole Sample	60	58	58	0.3148	**69.31**

Three of the samples (one, two and four) were superficial, approximately 10 mm in thickness, which only sampled the outer layer of the blubber. Sample five was almost full thickness and sample six, the only full thickness sample, was divided into three equal sections for individual analysis. Although there was no discernible visual stratification in these samples, Hooker *et al.* (2001) reported moderate fatty-acid stratification in blubber samples collected from this species that was less pronounced than that seen in small cetaceans. Total lipid content ranged from between 43.2% to 76.55%, with the superficial samples having the lowest lipid levels. In general as in the other species analyzed, the middle and inner layers had the highest levels.

Discussion and Recommendations for Future Research

These results demonstrate that full thickness samples are required wherever possible as samples collected only from the outer blubber layer would have underestimated the body

condition of this animal. The two deepest samples (five and six), using a 60mm biopsy tip, gave very comparable total lipid levels for the entire samples of between 68-69%. Clearly, this research could be improved upon with more samples, but it does indicate that a biopsy depth of 60mm is preferred to apply this method.

Sub-Project 3: Total Lipid Levels in Blubber Samples from Humpback Whales

Specified Detailed Methods

Superficial blubber biopsy samples (n=9) were collected from humpback whales in the St Lawrence estuary, Canada, during summer 2011. These animals were the same as those on which the 3MPD3GT tags were deployed.

Samples were divided longitudinally to allow hormone analysis to also be carried out on the same samples (see below). Total lipid content was determined using the same method as described above. The total mass of each sample portion was relatively small, ranging from 0.09g to 0.24g which unfortunately did not allow for lipid determination in duplicate as would be the recommended approach.

In addition, a sample of blubber from a stranded humpback whale (Brax) which was found on Anticosti Island in 2010 was analyzed for lipid content. It was a well-known female, first seen in 1982, so she was at least 30 years old. She was last seen with a calf in 2009 and hence was at the end of lactation by mid-November when she was found dead. The blubber sample was a full thickness sample of 109mm total depth. Duplicate subsamples were taken from the inner, middle and outer layers of the sample to investigate the variation with depth as in the previous study on stranded odontocetes (sub-project 1).

Results and Outcomes

The results of the remote biopsy samples are given in Table 9. There was quite a lot of individual variation with blubber lipid content ranging from 31.5% to 62.9%. One animal (Meduse) was sampled twice during the season in July and again in September during which time his blubber lipid had increased from 52.6% to 62.9%.

Table 9*. Total lipid content in remote blubber biopsy samples from tagged humpback whales. NS = not sampled*

Date	Animal	Name	Sex	Tag serial number	Lipid (%)
21/07/2011	H584	Manta	F	3745	NS
22/07/2011	H607	Meduse	M	3745	52.60
25/07/2011	H671	Pythagore	M	87666	46.50
25/07/2011	H686	Not named	F	3745	62.30
26/07/2011	H698	Not named	M	87666	56.40
26/07/2011	H731	Not named	F	3745	37.60
27/07/2011	H228	Gronier	F	3745	NS
28/07/2011	H584	Manta	F	87666	NS
19/08/2011	H707	Calanus	M	57537	NS
28/08/2011	H755	Not named	M	57537	56.30

Date	Animal	Name	Sex	Tag serial number	Lipid (%)
01/09/2011	H607	Meduse	M	57537	62.90
04/09/2011	H002	Splish	F	57537	31.50
18/09/2011	H405	Barbillion	M	63607	43.40
19/09/2011	H489	Not named	Unknown	63607	NS

The total lipid content for each duplicated subsample and the overall average percentage of lipid for each layer from the stranded humpback whale are show in Table 10.

Table 10. *Total blubber lipid content in the stranded humpback whale blubber sample. Numbers in parenthesis are duplicates.*

Blubber Sample	% Lipid
Outer Layer (1)	68.9
Outer Layer (2)	73.1
Mean Outer Layer (0-24mm)	**71.0**
Middle Layer (1)	51.9
Middle Layer (2)	54.8
Mean Middle Layer (24-75mm)	**53.3**
Inner Layer (1)	20.0
Inner Layer (2)	22.7
Mean Inner Layer (75-109mm)	**21.4**

Discussion and Recommendations for Future Research

The outer blubber layer had the highest lipid content in the stranded humpback whale. This is in contrast to the small odontocetes sampled but is in line with the findings of Elfes (2008) who also found the highest level of lipid in the outer layer of a blubber sample from a stranded humpback whale. This may indicate that relatively shallow biopsy samples can be used to quantify lipid content in humpback whales, however, a larger sample size is required to confirm this initial result and collection of deeper biopsy samples is recommended. For this reason, further work should concentrate on species-specific differences in blubber stratification to determine the representativeness of shallower biopsy samples.

Two 100mm biopsy samples were collected from humpback whales tagged in the Norway 3S study, which could be useful to further evaluate the depth-stratification of lipids in the blubber of humpback whales. However, due to delays with Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) permit clearances, those samples were not analyzed in time for this final report. The reason for the delays in obtaining the export permits from Norwegian CITES office were unknown but were protracted and took much longer than was anticipated. This also resulted in delays in obtaining matching import permits from the UK which then coincided with periods when our Norwegian collaborators were away in the field. We now have an export permit for these samples and will hopefully be receiving them shortly for analysis in the near future.

3) Foraging Effort, Energetic Performance, and Anti-Predator Behavior

Use the high-resolution tag data to quantify that individual's foraging effort, energetic performance, and anti-predator behaviors.

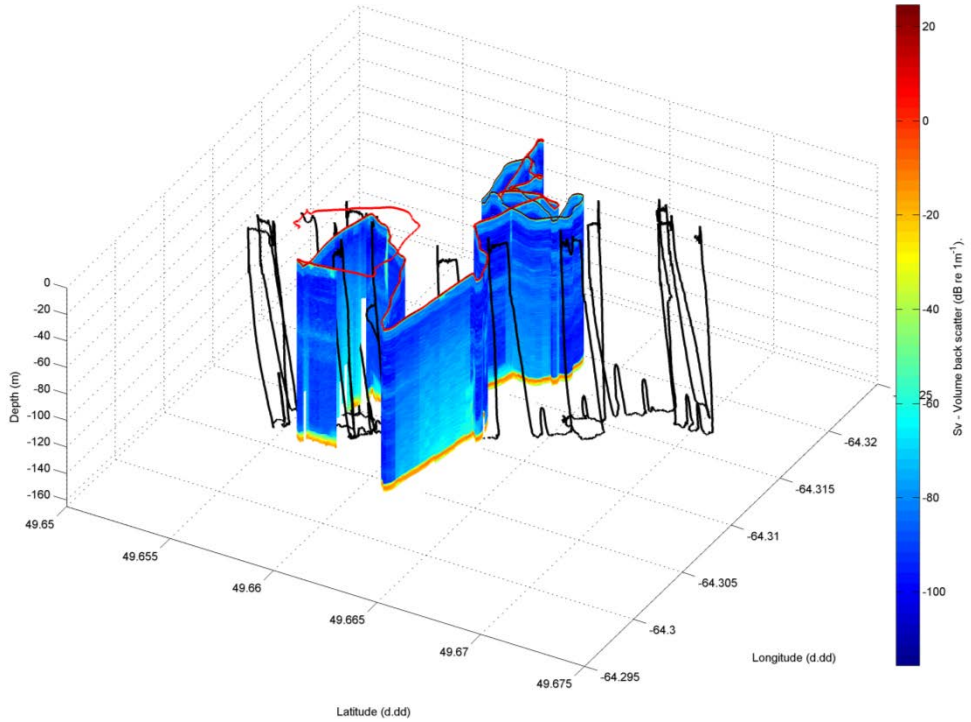
Specified Detailed Methods

The approach here is to use the record on the tag itself to identify the relevant behavioral patterns. Supporting environmental measurements can be made of the prey field. Therefore, this set of measurements is considered low-risk for the overall project because the tag data itself provides key metrics of foraging behavior (Watwood *et al*., 2006), energetic performance (Miller *et al*., 2004; 2010) and anti-predator behaviors (Tyack *et al*., 2006).

Results and Discussion

For the humpback whale, there is a clear indication of lunge-feeding within the tag record (see Figures 4 & 5), and indications of bottom feeding (Figure 17).

Figure 17. *Three-dimensional movement track of a humpback whale (black line) reconstructed from 3MPD3GT logger data. The ribbon plot shows volume backscatter data from a simultaneous echo-sounder survey. Note the whale was consistently making dives to the sea floor where it was likely bottom feeding on sand-lance.*



For bottlenose whales, deep diving behavior clearly represents foraging for this species. Table 11 summarizes the dive statistics of the three bottlenose whales in this study. Key foraging effort parameters include the % of time foraging at depth (>1000m). Energetic parameters include inter-deep-dive interval and breathing times (not shown here, but clear in the 3MPD3GT data records). Ascent pitch and rate are considered to be anti-predator behaviors (Tyack *et al*., 2006).

Table 11. *Diving statistics of the Northern Bottlenose whales tagged in this study.*

	Northern Bottlenose Whale Data		
	07-Aug	**08-Aug**	**11-Aug**
% of time <20m	58.9	57.1	62.5
% of time 20-100m	9.9	9.9	4.7
% of time 100-500m	8.7	7	7.5
% of time 500-1000m	7.6	6.6	10
% of time >1000m	14.9	19.4	15.3
surfacing duration (min)	9.6 ± 15.4	8.4 ± 5.8	10.6 ± 15.7
# of surfacing periods	(N = 30)	(N = 10)	(N = 27)
Number of deep dives	3	1	3
Deep dive depth (m)	1640 ± 23	1625	1500 ± 86
Deep dive duration (min)	46 ± 7	49	51 ± 4
Inter-deep-dive interval (min)	138 ± 157	-	50 ± 30
Deep dive descent rate (m/s)	1.8 ± 0.1	1.8	1.7 ± 0.2
Deep dive ascent rate (m/s)	-1.4 ± 0.1	-1.4	-1.1 ± 0.3
Deep dive descent pitch (deg.)	-60.3 ± 2.6	-66.2	-59.6 ± 9.0
Deep dive ascent pitch (deg.)	72.8 ± 8.2	63.5	49.5 ± 20.3
Time at depth >85% max (min)	13 ± 0.7	13	13 ± 1
% of dive > 85% max	33 ± 14	41	21 ± 11

For bottlenose whales, there might be a benefit to use the acoustic-recording DTAG, rather than the 3MPD3GT, as it records echolocation search and prey-capture sounds produced by the whale. However, the potential benefit of the acoustic sensor in the DTAG needs to be balanced against the cost of not having a speed sensor. However, Miller *et al*. (2004) was able to estimate body density of deep-diving sperm whales using the DTAG. Both types of tags should be considered for use in the full study.

4) Playback of Killer Whale Calls

Simulate predator presence via playback of killer whale calls.

Results and Discussion

Miller has been leading a study playing back killer whale (*orca*) sounds to heterospecific cetaceans as part of the 3S study of the effects of sonar on cetaceans in Norway. Prior to 2011, five full experiments have been conducted with both long-finned and sperm whales.

During the 3S-11 sonar trial in Norway, playback experiments were performed on three Humpback whales (Table 12). Each experiment of killer whale playbacks was performed from a Lubell speaker deployed from a tag boat and required roughly 1.5 hours. Two sound stimuli were played back in random order; 15 min noise or 15 min Killer whale sounds, with 30 min between exposures. The broad band noise signal was used as a negative control. The signal is a sequence of background noise selected from previous recordings, amplified up to get the average root mean square (RMS) power equal to the Killer whale stimulus, and repeated until getting the same duration as the stimulus (15 min). The Killer whale

vocalizations used was a recording from transient mammal-feeding Killer whales. It was recorded in 2005 in a behavioral context of foraging (DTAG acoustic recordings). All acoustic signals have a similar average RMS power (roughly -30dB) and duration of 15 min. To avoid pseudoreplication, we used three different sets of killer whales stimuli and three different noise stimuli. For all experiments, playback started at a distance of 800 m (estimated) at an angle of around 90° from the direction of travel of the focal animal.

Table 12. *Summary of the orca playback experiments performed with humpback whales.*

	Date and time of playback session			**Acoustic signals & comments on responses**	
	Date	*Time of Start (GMT)*	*Time of End (GMT)*	*Acoustic signals*	*Comments*
Humpback whale	06 June	11:40:40	11:56:51	1- KW 2- Noise	Changed direction in response to killer whale sounds. No visible response to noise.
Humpback whale	09 June	12:14:48	12:30:13	1- KW 2- Noise	Changed direction in response to killer whale sounds. No visible response to noise.
Humpback whale	15 June	12:13:00	12:29:00	1- KW 2- Noise	Changed direction in response to killer whale sounds. No visible response to noise.

With Northern bottlenose whales in the Gully, we were unable to consistently track tagged animals, which made it difficult to place the playback speaker correctly and visually monitor behavioral responses to killer whale calls. We therefore did not conduct any killer whale playbacks. Tracking was poor due to low tag placement, an issue which will be resolved using a 90° tag attachment system to place tags higher on the back.

Given the track-record of success conducting playbacks, this activity is considered low-risk.

5) Assessment of Pregnancy Status

For female subjects, evaluated methods to determine the subject's reproductive status at the time as either pregnant, nursing, or neither. Assessment of pregnancy accomplished using analysis of hormones (progesterone) collected in biopsy samples, and in blow expirate. Nursing status was assessed by observation of calf presence or absence at sea, and estimates of calf size when present. Animals are considered nursing if they are female and seen closely associated with a calf-sized animal.

- Visual monitoring for calf presence/absence was successful, and none of the study subjects was determined to be nursing as no calf was present in the group.
- We had no opportunity to measure calves using laser-photogrammetry, but visual classification of size is effective to identify calves. More work on techniques to measure animal length would be needed to measure calf length accurately enough to estimate time from birth.

5.1 Hormone Analysis of Blubber

Key Findings: Progesterone concentrations in blubber biopsy samples as indicators of pregnancy

- Progesterone concentrations in marine mammal blubber samples can be determined using a commercially available enzyme linked immunosorbent assay (ELISA). Very high levels were found in pregnant harbor seal blubber biopsy samples in a verification trial.
- Low levels of progesterone were found in blubber samples from a stranded female northern bottlenose whale suggested the animal was either not pregnant or was lactating at the time of sampling.
- Hormone levels were lower in the superficial layer than in the full blubber depth, suggesting for the Northern bottlenose whale shallow biopsy samples might underestimate the total blubber concentration. However, this has shown it is possible to use this approach as an indicator in this species.
- Similar results were obtained for the humpback whale where stratification in the blubber affected the concentration of progesterone in the various layers. However, one biopsy sample from a mature female had a high level of progesterone (~180 ng/g) indicative of pregnancy.

Background

Reproductive hormone levels, particularly progesterone have been measured in cetacean blubber samples, from various species, as indicators of pregnancy status (Mansour *et al*. 2002. Kellar *et al*. 2006). In samples collected from minke whales (*Balaenoptera acutorostrata*) where pregnancy status was confirmed post mortem, progesterone concentrations were significantly higher in pregnant females than in non-pregnant females (mean ± SE 133 ±23 ng/g blubber of pregnant compared with 1.95 ± 0.3 ng/g in non-pregnant females, $p<0.0001$) (Mansour *et al.* 2002). Similarly (Kellar *et al*. 2006) found that progesterone concentrations in three delphinid species were higher in pregnant (132-415 ng/g) than non-pregnant and immature animals (0.92-48.2 ng/g). Perez *et al*. (2011) were the first to test this method of pregnancy detection on free-ranging bottlenose dolphins and long-finned pilot whales. They found that mean progesterone levels in two pregnant animals were nine times higher than the non-pregnant animals with no overlap (~17 to 78 ng/g in pregnant animals compared with ~2 to 13 ng/g in non-pregnant animals).

Sub-Project 1: Progesterone Verification in Harbor Seal Blubber and Plasma

Specific Detailed Methods

In order to verify this method in our laboratory, blubber samples collected from potentially pregnant harbor seals (*Phoca vitulina*), and a male control were analyzed. Hormones were extracted following the method published by Kellar *et al.* (2006) and progesterone levels measured using a commercially available ELISA kit (DRG International Inc., USA, EIA-

1561). Blubber biopsy samples and blood plasma samples were collected simultaneously from live captured harbor seals from the UK during the breeding season (mid-June - early July) and stored at -20^{o}C. Samples were homogenized in 1000µl ethanol. Homogenates were centrifuged at 3,000 *rcf* for 10 min and the supernatants collected. These were evaporated under compressed air while incubating at 25^{o}C. Two milliliters of ethanol : acetone (4:1) were added to the residue and after vortexing and centrifugation, the solution was again evaporated. 1ml diethyl ether was added and after similar evaporation steps, 1ml of acetonitrile was added. Then 1ml of hexane was added, vortexed and centrifuged for 20 minutes. The solvents formed two immiscible layers with hexane on top. The acetonitrile layer was collected and re-extracted with 1ml of hexane, centrifuged for 20 minutes and the final acetonitrile layer aspirated and evaporated. The final residue was centrifuged and re-dissolved in 500µl phosphate buffered saline (pH 7.5) containing bovine γ globulin and mixed thoroughly for 15 minutes. Progesterone levels were then measured according to the ELISA kit instructions with a standard curve ranging between 0 and 40ng/ml. Samples were analyzed in duplicate and the mean level reported as progesterone concentration per wet weight of the sub-sample. Plasma samples from the same individuals were run unextracted.

Results and Outcomes

The results of all the progesterone analyses using both a linear log-transformed fit to the standard curve and a four parameter logistic regression model fit to determine the method precision at the upper and lower limits is shown in Table 13. The second method does not allow extrapolation beyond the upper standard. Although the linear method allows estimation above the range, the absolute concentrations calculated may be overestimated. However, for the purposes of this study both methods allow us to investigate the relative differences in concentrations of hormone in animals collected just prior to the harbor seal pupping season.

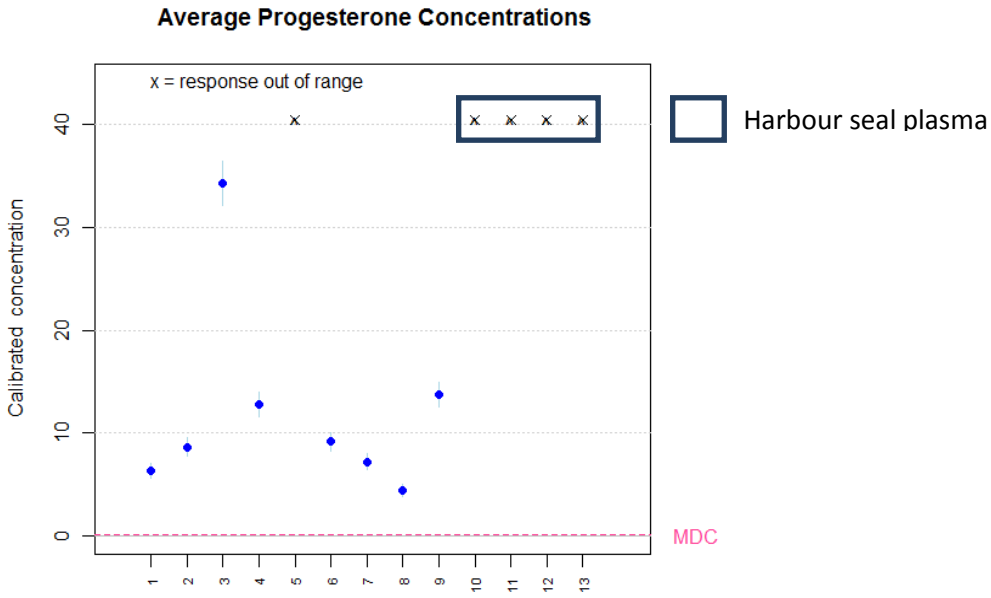
The average concentration of blubber progesterone for the duplicate samples calculated from the four parameter logistic curve and their standard errors are shown in Figure 18. The calibrated concentration on the y axis is the concentration measured in the extracts (ng/mL) which are then converted to concentration in wet weight of blubber based on the size of sample used in the analysis.

The progesterone concentration in the male sample was the lowest, as was to be expected. Three females had very high concentrations in their blubber (>200ng/g) one of which was well above the limit of detection (LOD) using the four parameter logistic standard curve. These results are in line with our knowledge of the timing of the pupping season for the regions where these animals were sampled. Those sampled in May were captured in the southeast of Scotland where the peak pupping is around mid-July. Those sampled in June were captured in Orkney, North Scotland where peak pupping is slightly earlier, around late June early July. Unfortunately we have no data on whether these animals then produced pups. However, as a surrogate for pregnancy status axillary girth was used to further investigate the relevance of the blubber concentrations.

Table 13. *Progesterone concentrations in harbor seal blubber biopsy samples (full thickness) and matched plasma samples. >LOD = above limit of detection.*

			Linear Model Standard Curve Calculations		Four Parameter Model Standard Curve Calculations	
Blubber Samples	**Sex**	**Date**	**Extract Progesterone (ng/mL)**	**Blubber Progesterone (ng/g)**	**Extract Progesterone (ng/mL)**	**Blubber Progesterone (ng/g)**
***Pv* 56312**	Male	17/03/2006	3.55	25.06	14.30	45.42
***Pv* 49804**	Female	09/05/2008	7.75	69.89	38.82	78.39
***Pv* 49805**	Female	09/05/2008	39.87	311.24	175.56	268.62
***Pv* 58520**	Female	09/05/2008	8.11	77.92	43.01	124.45
***Pv* 58522**	Female	09/05/2008	303.93	2883.54	>LOD	>LOD
***Pv* 59028**	Female	10/06/2009	8.26	117.07	62.67	130.45
***Pv* 59029**	Female	10/06/2009	6.28	276.52	141.40	318.50
Plasma Samples			**Plasma Progesterone (ng/mL)**		**Plasma Progesterone (ng/mL)**	
***Pv* 49804**	Female	09/05/2008	80.83		>LOD	
***Pv* 49805**	Female	09/05/2008	190.83		>LOD	
***Pv* 58520**	Female	09/05/2008	133.56		>LOD	
***Pv* 58522**	Female	09/05/2008	217.18		>LOD	

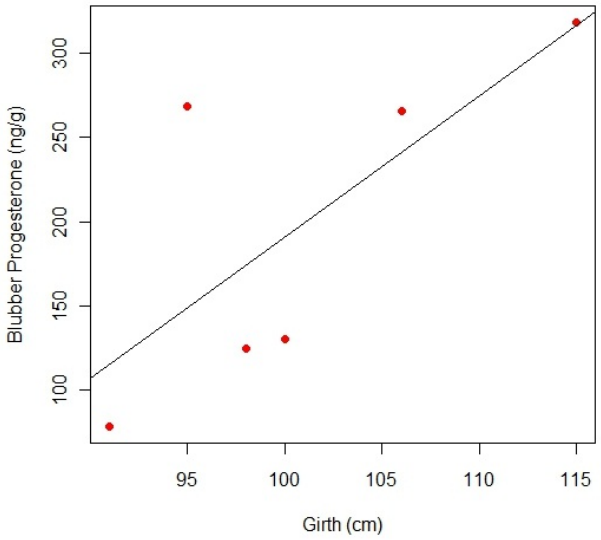
Figure 18. *Average concentration of progesterone in each harbor seal blubber extract or plasma sample from the duplicates analyzed by ELISA. The vertical bars indicate the standard errors and MDC = minimum detectable concentration*



The relationship between axillary girth and blubber progesterone is shown in Fig. 19. The sample >LOD was assigned a value equivalent to the maximum detectable concentration of 40ng/mL (= 266ng/g based on a sample size of 0.15g). There was a positive linear

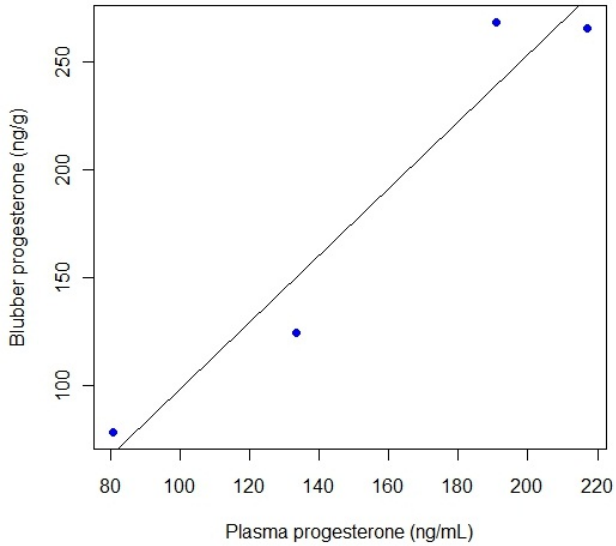
relationship but this was not significant at the 5% level (p=0.098, R^2=0.402) largely because one animal appeared to be an outlier.

Figure 19. *Relationship between axillary girth and blubber progesterone in harbor seals.*



As another surrogate for pregnancy status, the relationship between plasma progesterone levels (the standard matrix in which progesterone levels are measured in mammals) and blubber progesterone levels was investigated. All the plasma sample concentrations of progesterone were above the LOD using the four parameter logistic standard curve. For this reason, to enable us to obtain absolute plasma progesterone values for comparison, estimates were calculated from the log-transformed linear standard curve. While this approach is not ideal as it may overestimate the progesterone concentrations in the samples, constraints on time and resources limited the possibilities of performing further analyses. In future studies it would be possible to estimate the true concentration by diluting the samples with an appropriate ELISA buffer and re-analyzing the diluted extracts until they lie within the detection range of the standard curve generated by the four parameter model. There was a significant positive linear relationship between the concentrations measured in the blubber and those measured in the plasma (Figure 20. p=0.0326, R^2=0.904). The slope of the line is 1.55 indicating that the concentrations in the two matrices are almost in equilibrium.

Figure 20. *Linear relationship between blubber and plasma progesterone concentrations in matched samples from harbor seals.*



Sub-Project 2: Progesterone Concentrations in Female Humpback Whale Biopsy Samples

Specified Detailed Methods

Progesterone levels were determined in four remote biopsy samples from the humpback whales sampled in the St Lawrence estuary, and one sample from the stranded whale. The latter was divided into outer, middle and inner layers to determine variability in concentrations through blubber depth.

Results and Outcomes

One male and two immature females had levels ranging from 14 to 70 ng/g. However the mature female had a level of 180 ng/g, which when compared to concentrations measured in other studies and species (Mansour *et al.* 2002. Kellar *et al.* 2006) is indicative of pregnancy. The results are shown in Table 14. Two of the samples were very small which may mean the concentrations are less accurate. The biopsy samples ranged from 0.052 to 0.178g whereas the full depth samples from the stranded animal were between 0.132 and 0.17g. The method published by Kellar *et al.* (2006) suggests that sample mass should be between 0.1 and 0.2g. In addition there was considerable variation in the concentrations measured between the layers in the full depth sample. The concentration in the middle layer was less than half that seen in the outer or inner layers.

Table 14. *Concentrations of progesterone in the blubber of stranded and live biopsied humpback whales.*

Animal	Name	Sex	Blubber Progesterone (ng/g)	Notes
H671	Pythagore	M	57.77	Very small blubber sample.
H686	Not Named	F	69.05	Immature female. Very small blubber sample.
H731	Not Named	F	14.11	Immature female.
H002	Splish	F	**180.07**	Mature female. Probably pregnant.
Brax	Outer Layer	F	28.28	
	Middle Layer		10.43	
	Inner Layer		29.58	
	Average		**22.76**	

Sub-Project 3 : Progesterone Concentrations in a Female Northern Bottlenose Whale

Specific Detailed Methods

The remaining vertical half from two of the blubber biopsy samples collected from the stranded Northern bottlenose whale and previously analyzed for lipid content were analyzed for progesterone content. Samples used were the outer layer of five and whole depth of six.

Results and Outcomes

The results of the hormone analysis are given in Table 15. As can be seen the concentration in the superficial outer layer sample was only half that measured in the full depth sample. The concentrations found were also relatively low. In comparison to the harbor seals and the results of previous studies in cetaceans (Mansour *et al*. 2002. Kellar *et al*. 2006) this would suggest that this animal was either not pregnant or was lactating as this level is intermediate between the pregnant and non-pregnant levels described by Kellar *et al*. (2006). Unfortunately, a necropsy was not carried out on this animal and it is therefore not possible to confirm its reproductive status.

Table 15. *Blubber progesterone concentrations in blubber samples from a female Northern bottlenose whale (found dead October, 2009).*

Blubber Samples	Linear Model Standard Curve Calculations		Four Parameter Model Standard Curve Calculations	
	Extract Progesterone (ng/mL)	Blubber Progesterone (ng/g)	Extract Progesterone (ng/mL)	Blubber Progesterone (ng/g)
Outer layer	2.35	16.87	9.61	**32.03**
Full depth	8.90	40.76	24.82	**63.12**

Discussion and Recommendations for Future Research

As has been previously demonstrated (Mansour *et al*. 2002, Kellar *et al*. 2006, Perez *et al*. 2011) and now replicated in our laboratory, it is possible to use blubber progesterone levels in marine mammals to estimate reproductive status. We have demonstrated it is possible to detect progesterone levels in blubber samples from biopsied and stranded humpback whales using a commercially available ELISA kit. As for the other cetacean species, it would be important to verify the concentration that may be indicative of pregnancy for humpback whales specifically, but using the published approximate concentration of >~100ng/g, one sample was clearly indicative of pregnancy. But as with the blubber lipids, some stratification may be occurring since the concentration was so much lower in the middle compared to the other layers. This is a small sample size so these results should be treated as preliminary but they are very promising.

While we were unable to confirm whether the harbor seals gave birth, given the time of year, the high fecundity rate of 0.88 (0.86-0.90 95% C.I.) of harbor seals (Matthiopoulos *et al*. in press in J. Animal Ecology), the girth and weight of the animals, together with their high blubber and plasma progesterone levels, it is highly unlikely that these females were not pregnant. We are also unable to confirm whether the female humpback whale with high blubber progesterone levels was pregnant, but based on concentrations published in other studies of confirmed pregnant females (Mansour *et al*. 2002), it is likely that she was. With the high recapture rate of individuals in the Gulf of St Lawrence (Ramp *et al.* 2009, Ramp *et al.* 2010), the presence of a calf with this female in the following feeding season (summer 2012) could confirm these results.

Finally, the significant positive linear relationship between plasma and blubber progesterone concentrations in the harbor seals indicates that blubber progesterone levels measured using a commercially available ELISA kit are likely to be representative of the plasma levels, the usual matrix used for mammalian pregnancy diagnosis.

5.2 Hormone Analysis of Blow Expirate

Key Findings: Progesterone and steroid hormone concentrations in whale blow (expirate) samples

- The trials using liquid chromatography-mass spectrometry were not sufficiently sensitive to detect hormones in whale blow samples collected from various species.
- However, UPLC (ultra performance liquid chromatography, Waters Corporation) was able to achieve higher chromatographic resolution and sensitivity whereby both progesterone and cortisol levels were detectable in blow samples.
- Results are preliminary as the quantity of blow collected and degree of seawater contamination was not quantifiable using the current collection system. Further work to determine the quantity collected or some other form of normalization is required before valid comparisons between levels found in different samples can be made.

Specific Detailed Methods

1) Sample Collection

A recent study by (Hogg *et al*. 2009) reported that it was possible to detect reproductive hormones in whale blow samples using liquid chromatography-mass spectrometry (LC-MS). The method detailed in that study was therefore followed here, using samples collected from a variety of mysticete and odontocete whale species from Canada and Norway including humpback whale, sperm whale, long finned pilot whale and bottlenose whale. In some cases the animals were individually identifiable and sex could be determined (largely humpback whales sampled in the St Lawrence estuary) but in most cases it was not possible to determine the sex or maturity of the animal.

Whale blow samples were collected using the same method as described in (Hogg *et al*. 2009). Briefly, blow samples were collected remotely using inert nylon stockings stretched over a 5 inch embroidery ring attached to a pole (Fig. 21). The collection material was cleaned by sonication for 15 min in 100% acetonitrile and then for a further 15 min in Milli-Q water, changing the water every 5 min. Blow samples were stored on board the sampling vessel and during transportation to the laboratory in 5ml inhibitor (100 mM $MnCl_2$/100µg/ml amoxicillin/potassium clavulanate).

2) Hormone Extraction

Hormones were extracted using solid phase extraction (SPE) where samples were centrifuged at 3,000 *rcf* for 15 min to remove the sample and inhibitor from the stocking. Extracts were loaded onto the SPE cartridges that had been preconditioned with 20ml acetonitrile followed by 5ml deionized water. Samples were loaded at 5 ml/min and washed with 7.5ml deionized water to remove salts. Elution of the hormones was then carried out with 5 ml 100% acetonitrile and eluent dried under compressed air. Samples were reconstituted in 60ul 60% acetonitrile in preparation for LC-MS analysis. Since the LC-MS method can be used to investigate the presence of many different proteins simultaneously, the opportunity to investigate both progesterone and cortisol as a potential stress marker for future research was taken. Standards of progesterone and cortisol from 2 ng/µl to 100 ng/µl in 60% acetonitrile were also included.

Figure 21. *Collection of a whale blow-expirate sample from a humpback whale.*

3) Hormone analysis by LC-MS

Samples were first analyzed using a Micromass LCT^{TM} Liquid Chromatography-Electrospray Ionization-Mass Spectrometer (LC-ESI-MS) which is a high performance orthogonal acceleration reflecting TOF (time-of-flight) mass spectrometer coupled to a Waters 2795 high-performance liquid chromatography (HPLC) with an autosampler and photodiode array detector. Analyses were carried out at the Mass Spectrometry Facility, Biomedical Sciences Research Complex, University of St Andrews. Samples were ionized using a Zspray atmospheric pressure ionization (API) source and electrospray ionization. Ions generated in the ZSpray source are transferred to the TOF analyses via two differentially pumped ion guides and a hexapole. As ions travel from the pusher to the detector they are separated in mass according to their flight times, with ions of the highest mass to charge ratio (*m/z*) arriving later. An Xterra RP18 3.0 x 50mm liquid chromatography (LC) column was used with between 20 and 50µl injected volume depending on the trial. A gradient from 98% water 1% formic acid to 98% acetonitrile to 1% formic acid was carried out over 10 min (15 min total run time) at a flow of 0.2 ml/min.

Progesterone and cortisol standards of 2ng/µl were analyzed using an initial sample with a cone voltage of 20V. A gradient scan from 100 to 1000 mass-to charge ratios (*m/z*) in positive scan mode was carried out using the Waters Masslynx software. Extracted ion chromatograms were generated from the total ion chromatogram (TIC, as shown in panel three in Fig. 23) at 315.116 *m/z* for progesterone and 363.052 *m/z* for cortisol.

Clean peaks at 15.62 min for progesterone and 12.81 min for cortisol were seen (Fig. 22.). Subsequently, three extracted blow samples were run but corresponding progesterone and cortisol peaks were not seen in the spectrograms (Fig. 23). Therefore in order to determine the sensitivity of the method, six extracted blow samples were spiked with between 2 ng/µl and 50ng/µl cortisol and progesterone. Cortisol and progesterone specific peaks were just detectable in when 100ng was added to the sample. Samples spiked with 500ng (Fig. 24) did show detectable peaks but unfortunately these concentrations are well above what would be expected in a blow sample based on the studies by (Hogg *et al*. 2005, Hogg *et al*. 2009).

Figure 22. *Progesterone and cortisol standard mass spectrograms in SIM mode*

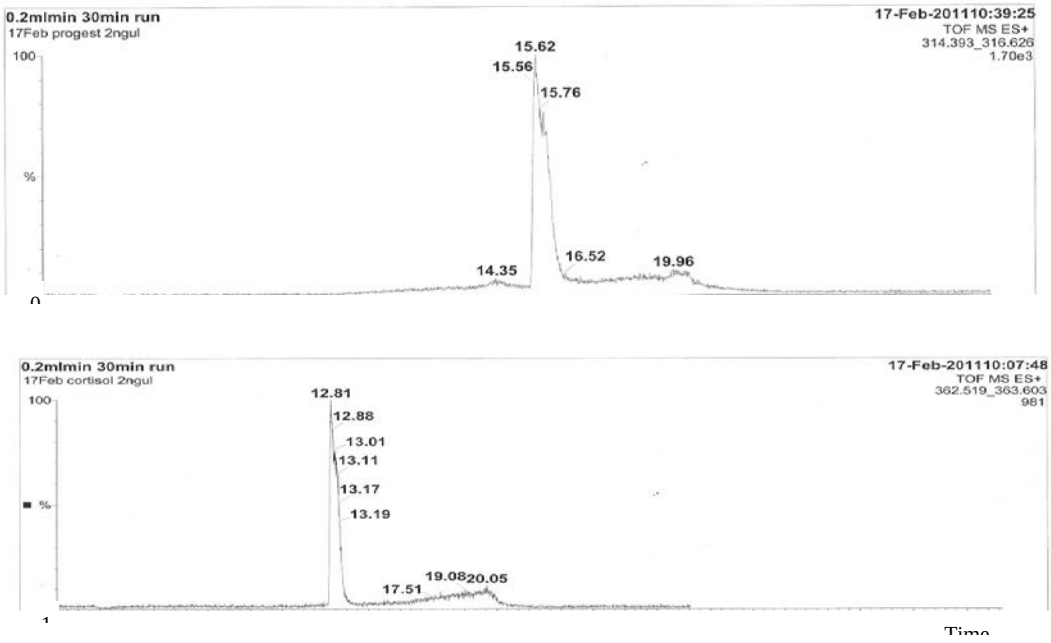


Figure 23. *Mass chromatogram of extracted humpback whale blow sample. Peaks at approximately (a) 15.6 min and (b) 12.8 should have been visible if the method was sufficiently sensitive or the proteins were present.*

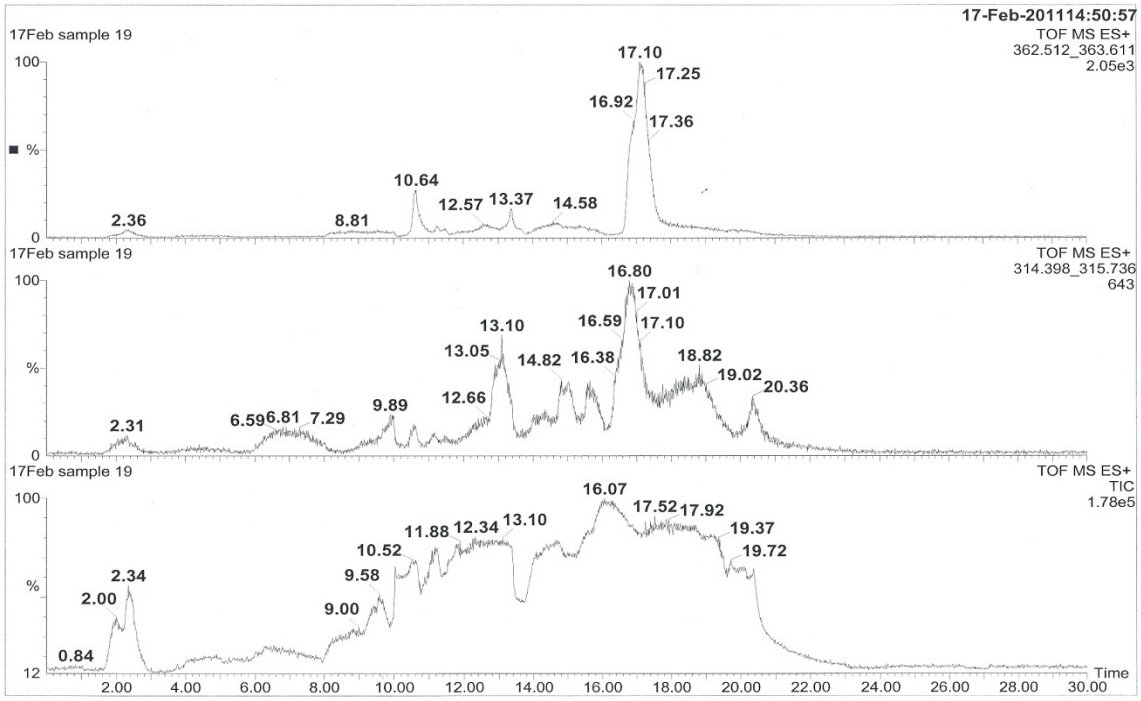
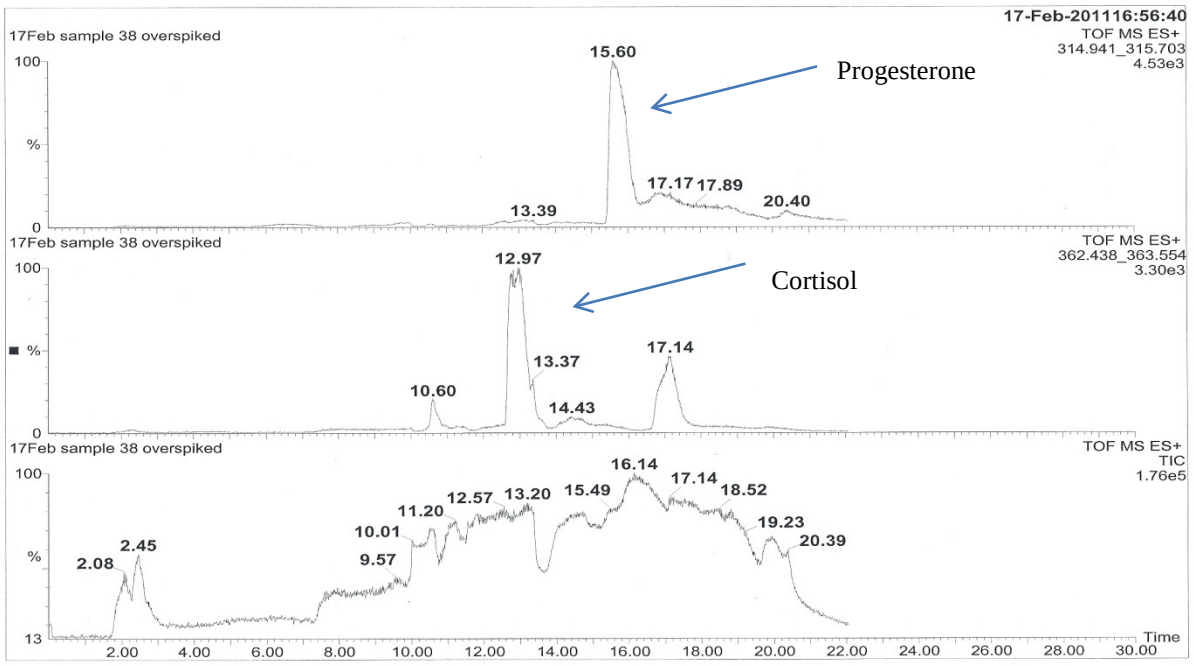


Figure 24. *Mass chromatogram of extracted humpback whale blow sample spiked with progesterone and cortisol. Peaks labeled (a) 15.6 min and (b) 12.97 min show the detection of these hormones.*



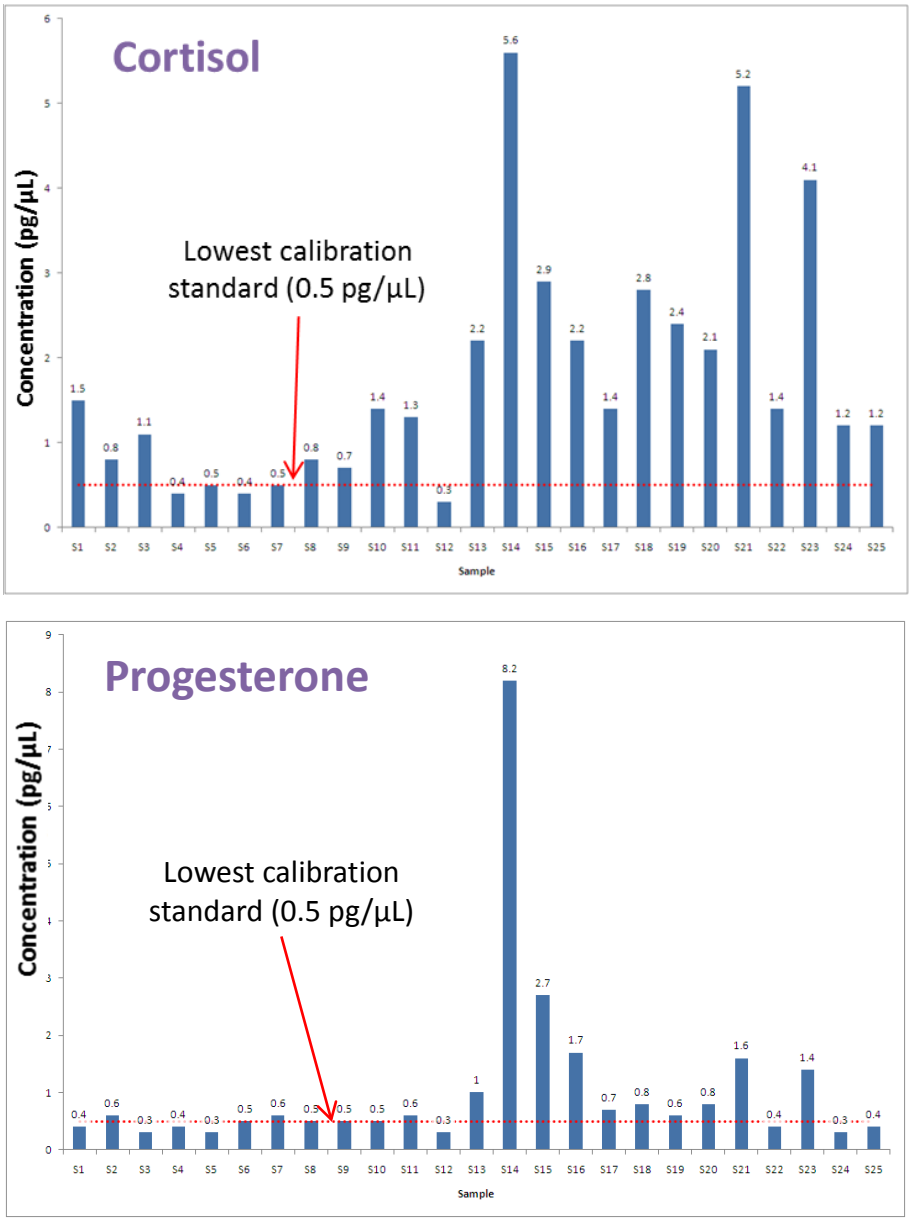
The opportunity then arose for additional samples to be analyzed using new UPLC technology manufactured by the Waters Corporation, Manchester, UK. Extracted blow samples and cortisol and progesterone standards as before were analyzed at the Waters Laboratories, Manchester using the Xevo TQ-S UPLC which is highly sensitive quadrupole instrument with photomultiplier tube detectors. A 5 point standard dilution series from 0.5 pg/µl to 50 pg/µl progesterone and cortisol were made up, and 25 whale blow samples were analyzed (Table 16). Experimental conditions used were water +0.5% formic acid mobile phase A, acetonitrile mobile phase B acquity BEH C. 2.1 x 50 mm column at 40^{o}C with a 0.6 ml/min flow rate. MS conditions were ESI+ ion source; capillary voltage 3.3 kV, 50V source offset, 150^{o}C source temperature 650^{o}C desolvation gas temperature 1000L/hr desolvation gas flow and 150 l/hr cone gas flow. Multiple reaction monitoring was used to increase selectivity and sensitivity. Further details of the method and instrument are available. Calibration curves were constructed which gave a linear response with an R^2 of 0.999 for both progesterone and cortisol and reproducibility was excellent with %RSD for both proteins being less than 2% for five replicates of a 5 pg/µl standard. As shown in Table 14 and Figure 25, cortisol was detected in 22 of 25 samples at a level higher than the lowest calibration point of 0.3 pg/µl. Progesterone was also detected in 13 samples higher than this point. Although most levels were very low, with values of between 0.3-2.7 pg/ µl, the highest level of 8.2 pg/µl was detected in a female humpback whale, which suggests she may have been pregnant. However, this female was seen again in 2011 without a calf suggesting that either she was not pregnant when sampled and the progesterone level measured here in the blow expirate, while higher than the rest, is not high enough to be indicative of pregnancy, or that she was pregnant and the calf died. Of the other two females sampled, one was lactating (when progesterone levels would be expected to be very low) and the other was last seen with a calf in 2008 and was seen without a calf in 2011. All the samples collected from males had very low levels.

Table 16. *Whale blow samples analyzed by UPLC. Quality code 1 = poor, 4 =excellent.*

No.	Species	Sex	Quality code	Cortisol pg/ul	Progesterone pg/ul
S1	**Humpback Whale**	CALF, UK	3	0.5	0.3
S2	**Humpback Whale**	F	2	0.8	0.5
S3	**Humpback Whale**	F	2	0.4	0.5
S4	**Humpback Whale**	F	3	5.6	8.2
S5	**Humpback Whale**	M	2	0.4	0.4
S6	**Humpback Whale**	M	3	1.5	0.4
S7	**Humpback Whale**	M	3	0.7	0.5
S8	**Humpback Whale**	M	3	1.4	0.5
S9	**Humpback Whale**	M	2	1.3	0.6
S10	**Humpback Whale**	M	3	0.8	0.6
S11	**Humpback Whale**	M	3	2.2	1
S12	**Humpback Whale**	UK	4	0.3	0.3
S13	**Humpback Whale**	UK	3	1.1	0.3
S14	**Humpback Whale**	UK	4	0.5	0.6
S15	**Humpback Whale**	UK	4	2.2	1.7
S16	**Humpback Whale**	UK	3	2.9	2.7
S17	**Long finned pilot whale**	CALF, UK	NR	5.2	1.5
S18	**Long finned pilot whale**	F	3	2.1	0.8
S19	**Long finned pilot whale**	UK	2	2.4	0.6
S20	**Northern Bottlenose whale**	UK	3	1.2	0.3
S21	**Northern Bottlenose whale**	UK	3	1.2	0.4
S22	**Sperm whale**	M	4	1.4	0.7
S23	**Sperm whale**	UK	2	2.8	0.8
S24	**Unknown**	UK	NR	1.4	0.4
S25	**Unknown**	UK	NR	4.1	1.4

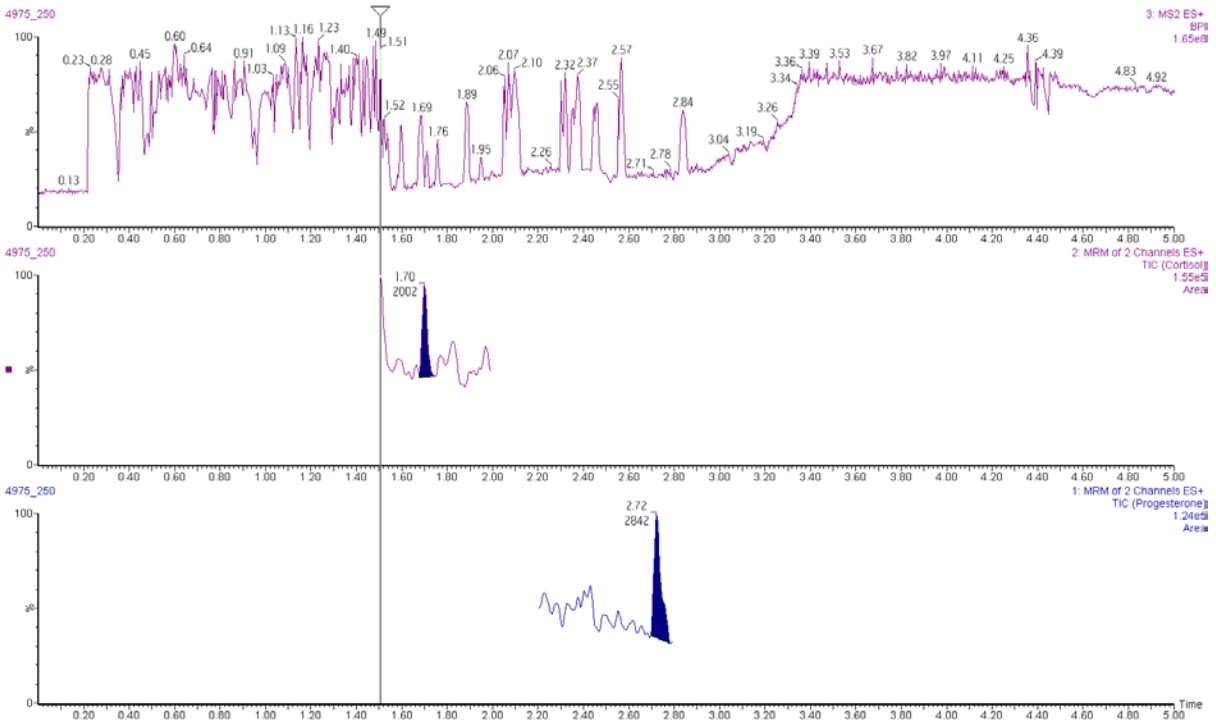
UK = unknown sex, NR = not recorded

Figure 25. *Concentrations of (a) cortisol and (b) progesterone in whale blow samples. NOTE: concentrations are pg/ul reconstituted extract and may therefore not be directly comparable.*



Full scan monitoring and simultaneous targeted analysis (known as Radar) can be carried out. This allows the analyst to see if there are other components in the same sample without having to run multiple injections. An example of the full scan spectrum showing both the cortisol and progesterone peaks identified is shown in Fig. 26.

Figure 26. *Xevo TQ-UPLC RADAR simultaneous targeted and full scan monitoring spectrum of a whale blow extract.*



Discussion and Recommendations for Future Research

This study has demonstrated that it is possible to detect and measure levels of steroid hormones, both progesterone and cortisol in whale blow samples. However, since the volume of blow collected is not known it is difficult to fully interpret the results. The highly sensitive UPLC method gave the most reliable results although duplicates of the same sample were not analyzed due to the limited volume of sample collected and extracted. Further work to determine the amount of blow collected, or normalization of the sample to another protein measured in the samples would greatly aid interpretation. In practically all mammalian species circulating progesterone increases linearly throughout gestation. This hormone is vital to ensure the functioning of the placenta and the maintenance of the pregnancy. Blood levels are therefore very high just prior to parturition. Although this has not been established for large cetaceans and studies in captive small odontocetes are limited (Sawyer-Steffan *et al*. 1983, Katsumata *et al*. 2006), results from blubber and fecal samples (Rolland *et al*. 2005, Kellar *et al*. 2006) that can clearly discriminate pregnant from non-pregnant cetaceans from high progesterone levels suggests the pregnancy hormone cycles are the same in these species.

6) Evaluation of Field Sites

As detailed above, the choice of target species is based upon the perceived benefit of studying the role of body nutritive condition on the behavior and reproduction in both a toothed and a baleen whale.

The humpback whale is an ideal candidate because it is known to fast during winter migrations, but humpback whales importantly display site fidelity to their feeding grounds (Stevick *et al*., 2006), so individual animals can be resighted over successive years. Adult and calf survival has been estimated in some populations for this species (Ramp *et al*., 2010a), so there is potential to assess the role of body condition in adult and calf survival. The field site in Mingan Island was an ideal base to carry out the shore-based research from a cost-perspective, and the long-term nature of their study is ideal for placing body condition within the context of vital rates. Alterations in the permit would be sought to allow deeper biopsy samples to be taken, and for killer whale playbacks to be conducted as planned in the study.

As a Ziphiid, Northern bottlenose whales are a species in a key taxonomic group of priority interest to DoD. As one of the few Ziphiid species to have been whaled commercially, relevant information on seasonality of calving and body length of pregnant females is available (Benjaminsen, 1972). Among the Ziphiids, Northern bottlenose are relatively well-studied, particularly the proposed study population in the Gully, Canada. They have been tagged with suction cup tags and a long-term photographic database already exists. One important vital rate which has not been studied in the Gully site is calf survival (Whitehead, personal communication). This is because young bottlenose whales are not sufficiently well marked to track individuals after they disassociate from their mothers. The permitting process to conduct this project in the Gully was intensive, but all relevant permissions were finally obtained from the authorities.

Permitting for Northern bottlenose and humpback research in Norway under the 3S project was obtained as part of the 3S study on the effects of noise on cetaceans. Compared to the sites in Canada, there has been little research on the target-species populations in Norway and no catalog of individuals exists. While certain aspects of the research might be accomplished in other locations than those used in the pilot study, the Gulf of St Lawrence for humpback whales and the Gully for Northern bottlenose whales appear to be well-suited to the requirements for this research project.

CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

The proposed work has many different research components, each of which entails a substantial level of complexity. In this limited-scope study, we have been able to successfully accomplish all of the proposed measurement or field activities, with at least one of the two target species (Table 17). This demonstrates our own ability to conduct the different components of the work, increasing the chances for success in the project. However, it was not possible to evaluate every single procedure for both subject species.

Table 17. *Outcome of risk-reduction limited-scope effort by task. Key measures are those that are primary metrics to be used in the full study. Cross measures are cross-validation techniques. Risk column shows perceived risk prior to the study, and after completion.*

Objective task	Method / sampling	Value to study	Risk before/after	Northern Bottlenose	Humpback whales
1) Measure body density	Glide kinematics from tag device	key	High / low (medium for humpback)	Successful in all cases	Not fully explored. Gases?
2a) Body shape/ dimensions	Photogrammetry	cross	High / High	Shape and body dimensions can be measured, not girth	
2b) % Lipid in blubber	Biopsy sample	cross	High / Low	Successful: 60-100cm depth Location must be consistent	
3) Foraging Effort & Anti-predator behaviors	Tag record	key	Low / Low	Relevant diving parameters quantified	Identified lunges and bottom feeding.
4) Anti-predator	*orca* playbacks	key	Medium / Low (medium for bottlenose)	Tag placement needs to be improved	Conducted three playbacks successfully
5) Identify reproductive status as pregnant, lactating, or resting	Biopsy sample (progesterone)	key	Med / Low	Status unconfirmed	one presumed pregnant
	Blow expirate (progesterone)	cross	High / Medium	Hormones found, but no reference is available	
	Observations of associated calf (lactating)	key	Low / Low	No calves were seen, but we were able to make the required observations	
6) Evaluate field sites	Field experience and evaluation	key	Permit risk reduced	No calf survival	Vital rates can be studied

Below we describe the conclusions and implications for each specific objective.

Measure body density using hydrodynamic analysis of tag data

Most critically for this study of body condition in free-ranging cetaceans, the breakthrough technique of measuring total body density from high-resolution tags has been successfully demonstrated in the Northern bottlenose whale. As envisaged in the initial proposal, the deep-diving behavior of the Ziphiidae is very well suited to measurement of body density because the effect of gases can be effectively ignored during deep glides (Biuw *et al*., 2003). We found, importantly, that the Northern bottlenose whale made substantial use of glides during both the descent and ascent phases of deep dives. Ascent from deep dives tended to be at steeper pitch angles than has previously been reported for Mesoplodon and Ziphius c. (Tyack *et al*., 2006). All of these aspects of the diving locomotion of the Northern bottlenose whale are ideal for measuring body density from the acceleration during glides, reflected in the narrow confidence intervals for our measures of body density. We were also able to estimate the diving lung volume of the tagged whales, using a fuller glide model and glides performed at shallower depth. Those results are novel, and are being prepared for submission to Frontiers in Aquatic Physiology.

In the limited duration of this study, we were not able to fully analyze the humpback whale data for body density. Detailed inspection of the data do indicate that whales make use of glide, but the shallower depth of the glides indicate that body density will need to be solved for simultaneously with diving lung volume (e.g., Miller *et al*., 2004).

Implications: From the perspective of risk-reduction, we feel there is little risk that we will not be able to measure the body density of beaked whales. There is still some risk that we will not be able to calculate body density for the baleen humpback whales as precisely as for the deep diving toothed whales. Therefore, we feel that research attempting to apply the glide method to estimate the body density of shallower-diving cetaceans is the appropriate next step in the development of this research.

Cross-validate the glide-derived measure of blubber lipid content

We made a substantial effort to evaluate the feasibility of photogrammetric and tissue-analysis approaches to quantify body condition. Not surprising, neither of these techniques by themselves are entirely satisfactory for quantifying the lipid stores of free-ranging cetaceans. Laser photogrammetry was successful in that we were able to use the method to make measurements from whales. Photographs taken from behind may be effective at revealing the state of fat stores in humpbacks. Critically, however, the key metric of body girth was not possible to measure from a small boat.

We conducted a series of analyses of blubber biopsies to evaluate our ability to measure the lipid concentration in the blubber, which may be a useful indicator of nutritive body condition. We were able to measure lipid concentration in biopsy samples taken manually or remotely using the pneumatic ARTS (Norway) or a cross-bow system (GSL). While the analysis outputs a number for lipid concentration, it is difficult to relate that number can be related to quantity of the body lipid store. The extrapolation to whole-body lipid store is complicated by the location and depth of the biopsy samples, in a fashion that seems to vary between species.

Implications: Cross-validation of body condition using visual assessment remains challenging. Body shape metrics, like that used by Bradford et al., (2012) are feasible with

humpback whales, but little is known about such an approach with bottlenose whales. Other existing approaches like aerial photogrammetry are very expensive and impractical for offshore species like beaked whales. It might be fruitful to explore new alternatives to viewing the whale from above, specifically to view it from below using a stereo underwater scanning sonar system. An underwater high-frequency sonar-based approach would enable the whole body to be viewed, and would reduce the requirement to be strictly oriented relative to the whale when measuring body dimensions.

We demonstrated the feasibility of measuring lipid content in blubber biopsy samples. The ARTS biopsy system was highly effective for collecting deep samples (up to 100 mm), and is therefore the recommended biopsy collection system. Our analyses of available samples suggested that 60 and 100mm depth sampling tips would be deep enough to account for most of the variation caused by blubber stratification in bottlenose and humpback whales, respectively. However, a sampling method that always collects the full blubber layer would be the best approach as it would provide an additional point estimate of the blubber thickness at the sampling location. However, permits for full-depth biopsy sampling may be difficult to obtain in some field locations. Indeed permitting complications led to our inability to collect biopsy samples from bottlenose whales. Prior to each data collection effort, it would therefore be valuable to update our sampling protocol to reflect the most recent information available about depth-stratification of lipids within blubber. Variation by location on the body is important, and standardization of biopsy sampling should therefore be a central goal of the research. Ideally, this should be a location which is commonly sampled, such as just below the dorsal fin.

Quantifying foraging effort, energetic status, and anti-predator behaviors

We predict that variation in body condition should be an important factor driving how individuals trade-off the need to feed with the risk of predation (McNamara & Houston, 1990). The tag records that we collected from both target species contain rich indicators of foraging effort, and patterns such as ascent rate that reflect anti-predator risk (Tyack *et al*., 2006). The 3MPD3GT tag is the ideal tool to measure body density, but suffers from a lack of an acoustic sensor which is helpful for describing the foraging and social calling behavior of subject animals.

Implications: Both tag types provide rich data enabling quantification of the foraging effort of both species. However, as there would be a benefit from the acoustic sensor on the DTAG with bottlenose whales, it is recommended that the research should explore the ability to measure body density using DTAGs as was previously done with sperm whales (Miller et al., 2004). They key factor in this evaluation is how steeply the animals ascend and descend during deep dives. Characteristics of Ziphiid diving behavior are considered likely to have an anti-predator function (Tyack et al., 2006), but it is not clear if/how humpback whales in a foraging area might alter their behavior to reduce predation risk.

Simulation of predator presence using playback of killer whale calls

To more fully explore the anti-predator behaviors, the research proposes to play the sounds of killer whales to the whale subjects. We successfully conducted those experiments with humpback whales in 2011 and have previously conducted orca sound playbacks to long finned pilot whales and sperm whales. It was not possible to trial playback of killer whale sounds with bottlenose whales as the tags were placed too low on the body of the whale for

effective very high frequency (VHF) tracking. Such tracking is critical to appropriately place the speaker relative to the position of the subject whale.

Implications: This approach is feasible and can be considered low risk for humpback whales using the current methods. Tag-attachment procedures for bottlenose whales need to be improved to consistently target tag locations high-enough on the body to enable effective VHF tracking of the tagged whale.

Determining female reproductive status (pregnant, lactating, or resting)

For reproductive status, we were able to demonstrate that we can measure the pregnancy hormone progesterone from blubber samples as has been previously documented to indicate pregnancy status (Mansour et al., 2002; Kellar et al., 2006; Perez et al., 2011), and can detect progesterone within blow expirate samples. In our study, one humpback whale showed clear indications of being pregnant from biopsy progesterone concentrations (Table 14) and one blow sample from a second female had elevated progesterone content (Figure 25). The second female was identified the following year without a calf, but the animal may still have been pregnant at the time of sampling if the fetus was aborted or the calf died during its first year of life. Though none of our study animals were seen travelling with a calf, indicating lactation status, we feel such observations are entirely feasible.

Implications: Measuring progesterone levels in blubber and detecting it within blow expirate appears to be a highly effective means of determining pregnancy status and should continue. However, the higher levels reported in blow samples are, at this stage, only a relative measure compared to all the other samples analyzed and have yet to be validated in terms of the volume of blow expirate collected. In addition, the relationship between the blow and blubber progesterone concentrations has yet to be established. Thus, we have a high degree of confidence that we will be able to link measurement of body composition to the sex and reproductive status of the subjects from biopsy samples, but more research is required to validate the blow-expirate method. Though no calves were seen associated with our subject whales, it was deemed highly feasible to identify cases when calves were travelling with the subject. However, identification of lactation status using hormone concentrations would be desirable and should be pursued if possible.

Evaluation of field sites

We have been able to accomplish most of the different components of the research, and in many cases, accomplish several of them with the same study subjects. However, it is important for this research that all of the key samples are collected from all subjects. Approachability of the research subjects is therefore important. Our limited-scope study has shown that both Northern bottlenose and humpback whales are relatively easy to approach and work with from a small vessel in the Gully and Gulf of St Lawrence field sites. We successfully deployed three tags to bottlenose whales in three days of workable weather, a good rate for beaked whales. Permits were obtained for all activities for both species, but some restrictions were put in place for our research with bottlenose whales in the Gully.

The most ambitious longer-term aspect of the proposed work is to ultimately tie in measures of body condition with indicators of reproductive success (Miller *et al*., 2011). This is best done in a study of a population with long re-sighting records. Unfortunately, though there is an identification catalogue for Northern bottlenose whales, it has proven difficult to measure

vital rates, particularly the key parameter of survival after weaning. However, the population of humpback whales successfully studied in this effort with MICS is a perfect example of a long-term study with high re-sighting rates of known individuals year after year.

Implications:

The evaluated field sites appear to be well-suited for the goals of the study. An appropriate study on how body condition varies with reproductive status and influences individual behavior is feasible with bottlenose and humpback whales. Body condition can be related to variation in vital rates in the GSL field site, if we are able to measure body density of humpback whales using the tag-based hydrodynamic method.

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